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Editor
ROY WALDO MINER

RECENT ADVANCES IN THE STUDY OF THE STRUCTURE, COMPOSITION, AND GROWTH OF MINERALIZED TISSUES

BY

ROY O. GREEP AND ALBERT E. SOBEL (*Conference Co-Chairmen*), W. D. ARMSTRONG, L. F. BÉLANGER, R. C. GREULICH, S. B. HENDRICKS, H. C. HODGE, C. P. LEBLOND, P. L. MUNSON, W. F. NEUMAN, R. A. ROBINSON, D. B. SCOTT, J. H. SHAW, R. F. SOGNNAES, M. V. STACK, O. R. TRAUTZ, M. L. WATSON, J. H. WEIKEL, JR., AND L. WILKINS

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RECENT ADVANCES IN THE STUDY OF THE STRUCTURE, COMPOSITION, AND GROWTH OF MINERALIZED TISSUES*

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Consulting Editor: ROY O. GREEP

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* This series of papers is the result of a conference on *Recent Advances in the Study of the Structure, Composition, and Growth of Mineralized Tissues* held by the Section of Biology of The New York Academy of Sciences, January 22 and 23, 1954.

INTRODUCTION

By Roy O. Greep
Harvard School of Dental Medicine, Boston, Mass.

The New York Academy of Sciences has held many conferences covering a wide range of biological topics, but I believe that the meeting on which this monograph is based marks the first time that the hard structures have had their day under this famed institution's sponsorship. It is a fitting occasion. The hard tissues have lent themselves admirably to exploration by some of the newer tools of science with the result that unusual scientific progress has been made in the elucidation of these structures. Penetrating study of the hard tissues by electron microscopy, X-ray diffraction, histochemistry, radiobiology, and microchemical and crystallographic methods has already carried us to units of structures that may be of ultimate character. Glimpses into the secret precincts of the molecular structure of these tissues have already been had.

As I have indicated, there has been an extraordinary resurgence of interest in the study of bones and teeth. This development has largely come about as a shift in attitude. By classical teachings of not too many years ago, the biological importance of bones did not extend far beyond their usefulness as a supporting tissue, as protection of vital organs, and as an aid to locomotion. The teeth, too, were important only for grasping or comminution of food, whether plant or prey. According to these precepts, bones and teeth were banished to the arid realm of the inanimate. True, the skeleton was but a crutch to the flesh and the teeth were grinding mills situated with great utilitarian appropriateness at the digestive portal. How different is the modern conception of these structures! For the bones and teeth are, indeed, dynamic structures which metabolize, adapt, and respond. Hard as they are, they are unstable and are constantly undergoing change. They also contribute with remarkable facility to the ionic equilibria of the body fluids. Recent demonstrations of the fact that bones function as a readily available reservoir of sodium is but a striking case in point. The field of hard tissue research is an active and exciting one.

The hard tissues of the body are interesting and important in another respect. They have a heritage in antiquity that has influenced profoundly the history of thought. Man's knowledge of the evolutionary emergence of today's Mammalia is based on imprints and fossilized remains of bones and teeth left in the geologic record of the earth's crust, the Piltdown man notwithstanding. Contemplation of the skeletal remains of animals that roamed the surface of the earth millions of years ago permits man to see himself and his present position in proper biological perspective. We see only the bony remains; but, in the mind's eye, these remains are swathed in flesh and blood. Why? Because, from present day forms, we have learned much of how the skeleton and teeth are molded to serve the needs of the organism. However, it is not that our bones and teeth stand the greater measure of chance at immortality that we gather here to consider their structural nature. Even if it should be shown that, in

crystal structure, the body minerals resemble one of the earth's minerals, we should not be able to produce artificially either bone or tooth, as these structures are products of a biological system. The blueprints for their formation are not available; they reside with other well-kept secrets of cellular activity.

The thoughts uppermost in the minds of your chairmen in arranging the conference on which this monograph is based, have been (1) to bring together experts who would paint as definitive a picture as possible of the present state of knowledge concerning the mineralized tissues; and, (2) to foster an exchange of ideas that may spotlight new paths for investigation or may coordinate the work being done at outposts along this expanding salient.

The general organization has been planned as follows: we shall first have a close look at the fine structure of the mineralized tissues; then we shall examine the nature of the substrate in which the mineral material is deposited. The relationship of the resulting crystals and the organic matrix will be pictured and, by radioactive tracers, the formation of teeth and bones will be outlined. The chemist and physicist will then open the chambers of ultrastructure where all formed matter is resolved into such universalities as atoms, energy bonds, and, lattices. Next, we shall examine metabolic, nutrient, and hormonal factors which influence the mineralization process. More difficult to assess, but of the quintessence in skeletal development and dental apposition, are those factors classed as constitutional. These factors also determine growth and growth potential. Involved here are heredity, hygiene, history of disease, food habits, and appetite and, therefore, mental health also. An unhappy child, by undereating or overeating, or by bizarre dietary habits, will foil the workings of an otherwise precise and reasonably predictable biological system.

The virtues of the multidiscipline conference are well known. This study brings together men from many disciplines, including chemists and physicists, anatomists and histochemists, physicians and dentists, and biologists and biochemists. These participants will appraise and criticize, laud and lament, compare and contrast, reason and deduce, speculate and stimulate.

As a final feature, we have asked certain of our colleagues to tell us, drawing upon the material presented and upon their own extensive experience, what new opportunities they see for future investigations, and what interesting possibilities they see for applying present knowledge to dental problems and to problems of skeletal growth and bone diseases. Finally, issues of more general nature will be discussed, and will furnish a rare opportunity to find wise and thoughtful answers to our problems.

Not only do we hope to find new ways to greater knowledge. We hope that zest and meaning will be added to our individual endeavors and will sharpen the direction of our attacks upon the major problems as they relate to health and human welfare.

MICROSTRUCTURE AND HISTOCHEMICAL CHARACTERISTICS OF THE MINERALIZED TISSUES*

By Reidar F. Sognnaes
Harvard School of Dental Medicine, Boston, Mass.

This presentation will not deal with bones, but with bone, as aptly defined by Weinmann and Sicher (1947); and not with teeth as organs, but with their mineralized tissues.

The discussion, primarily, will be concerned with histological and histochemical studies, currently pursued in our own and associated laboratories or reported in the literature during the last few years. Since it is difficult, however, to develop this subject adequately within the limits of two specific disciplines, a few pertinent references will be made to related studies, using other tools as a means to the same end: an introductory consideration of the structural basis for the biological behavior peculiar to skeletal and dental hard tissues.

(1) *Histomorphology*

The development of our knowledge about the histology of bones and teeth has been most comprehensively described and illustrated by Weidenreich (1930), Petersen (1930), and Lehner and Plenk (1936), respectively, in the second and third volumes of *Möllendorff's Handbuch der mikroskopische Anatomie des Menschen*. The morphological characteristics of bones and teeth were covered in great detail, but the two were not related to each other.

Petersen (1930) suggested a morphological division of bone into four structural orders, translated and summarized in abbreviated form in TABLE 1. At increasingly higher levels of observation, orders one to four are concerned with gross architectural, vascular, cellular, fibrous, amorphous, and crystalline components of bone. For obvious reasons, it was not attempted to fit the dental hard tissues into this organizational scheme. However, while bones and teeth are far apart with respect to morphology and evolutionary origin, there is in the mouth of higher animals a close interrelationship between development and function of all hard tissues: alveolar bone, cementum, dentin, and enamel. An attempt will be made here to treat the four mineralized tissues in parallel fashion with respect to form, composition, and function alike, and with a view to establishing similarities basic to all.

From a histomorphological point of view, there is relatively little descriptive material that, today, can be added to what has already become textbook information (Greep, 1954). For reasons that will become apparent below it is, nevertheless, of interest to make a direct comparison of the microscopic landmarks of the hard tissues at comparable magnification. FIGURES 1 to 9 illustrate the appearance of decalcified sections of alveolar bone, cementum, dentin,

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TABLE 1
STRUCTURAL ORDERS OF BONE
(Petersen, 1930)

Orders	Structural components
First	Spongiosa and compacta
Second	Haversian systems and lamellae
Third	Lacunae, osteocytes, and connective tissue fibers
Fourth	Amorphous ground substance and inorganic salts

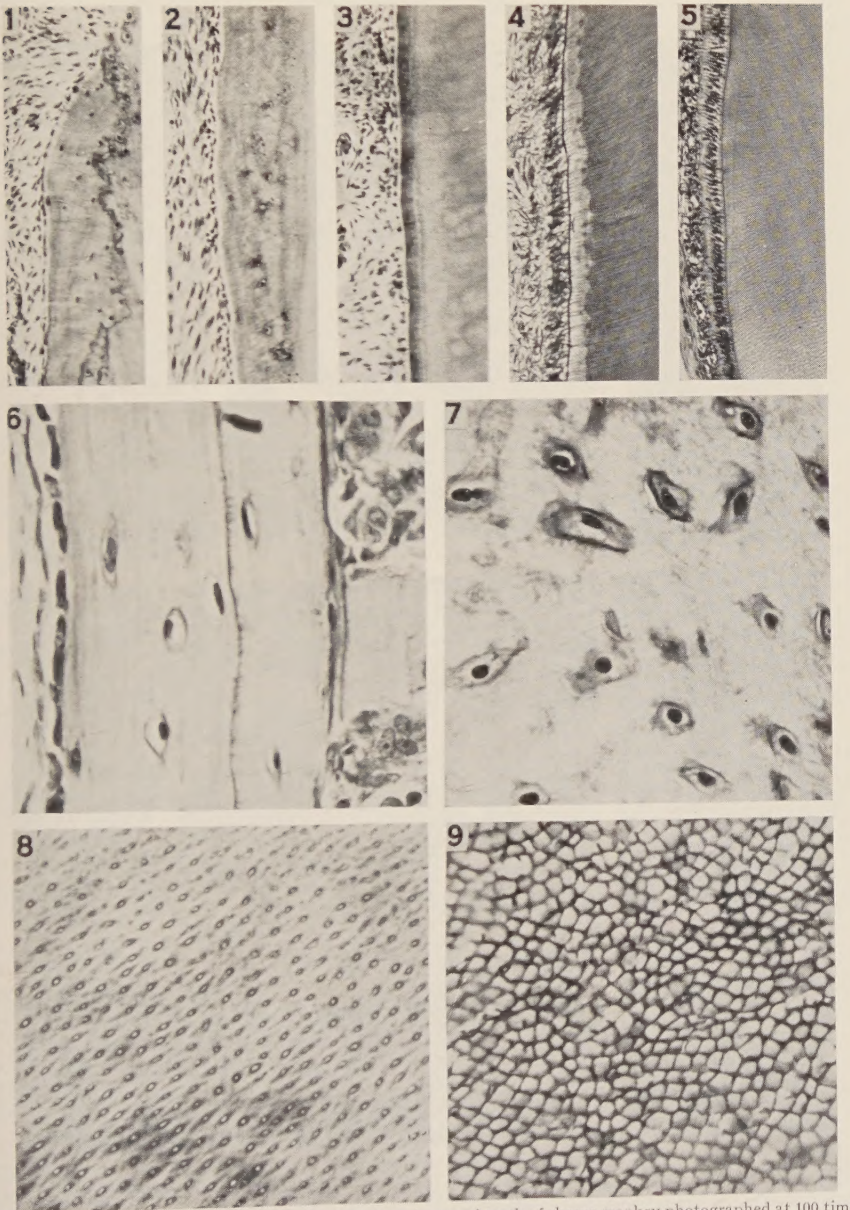
and enamel; FIGURES 1 to 5 examined at 100 times, and FIGURES 6 to 9 at 500 times.

At the lower magnification, the cellular components appear similar wherever they are adjacent to bone (FIGURE 1), to cellular or secondary cementum (FIGURE 2), and to acellular or primary cementum (FIGURE 3). At this magnification one cannot readily distinguish the uncalcified osteoid or cementoid layers. In dentin (FIGURE 4), on the contrary, there is normally a clearly distinguishable wide zone of uncalcified predentin, which persists throughout life. With appropriate stains, to be shown later, a similar distinction can be made between pre-enamel and enamel, but this distinction does not persist in the enamel of mature teeth. Furthermore, the cellular layers which cover the developing enamel (FIGURE 5) disintegrate when the tooth erupts.

At the higher magnification, there is a considerable similarity in the appearance of the internal cells of bone (FIGURE 6) and cellular cementum (FIGURE 7). In the case of bone, one normally encounters, in addition to the osteoblastic layer, a varying number of osteoclasts, two of which are seen at the right in FIGURE 6. These cells are readily observed in connection with the cellular remodeling of the alveolar bone during growth and in response to function and orthodontic tooth movement, whereas the adjacent bonelike cementum, for reasons that are not clear, is normally spared from such resorption, with one exception to be noted later.

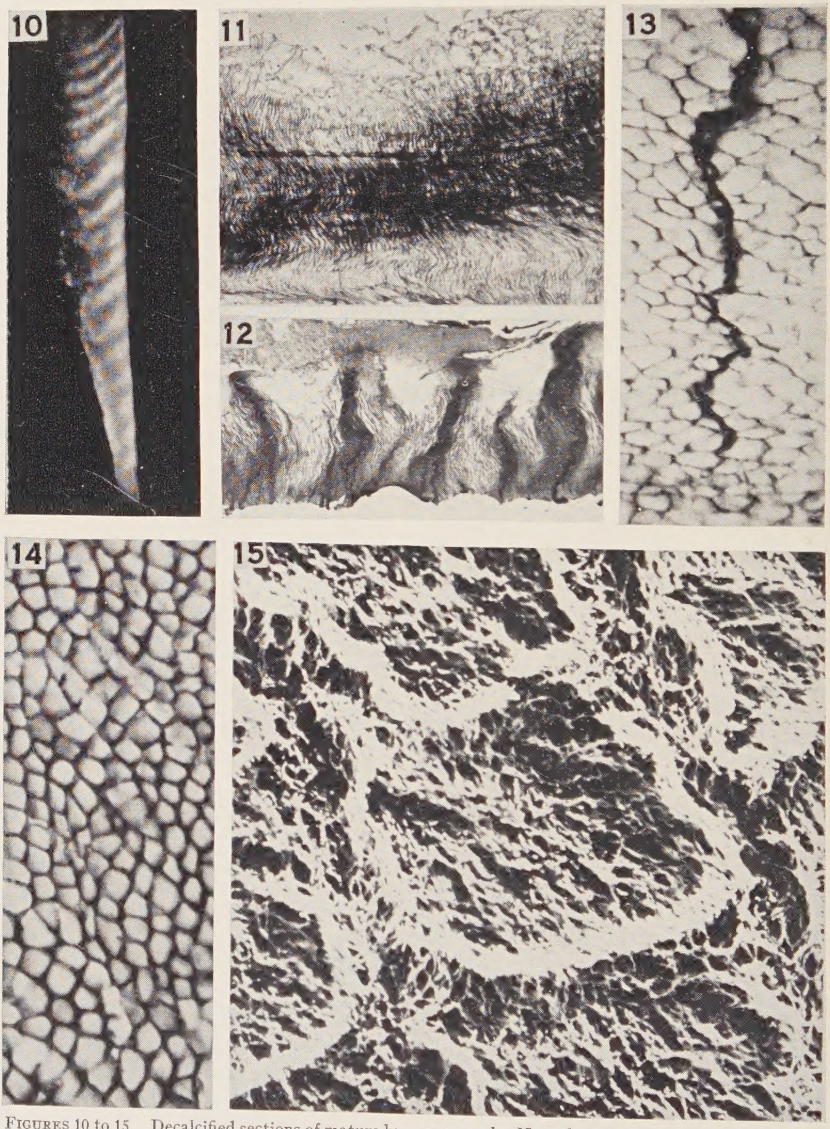
When dentin and enamel are studied at the higher magnification ($\times 500$), it is observed that neither structure contains any cells. Instead, the dentin (FIGURE 8) is permeated by a multitude of minute tubules which contain protoplasmatic extensions from the odontoblasts of the pulp. The enamel (FIGURE 9) is entirely prismatic in nature and devoid of cellular and vascular elements. The prisms are surrounded by a honeycomb arrangement of organic matter which, at this magnification, appears concentrated between or in the periphery of the prisms. Due to these minute dimensions, the ordinary microscope leaves much to be desired for a precise examination of the content of the dentinal tubules, the enamel prisms, and, especially, the prism sheaths and interprismatic regions.

In addition to the small dimensions involved, there are considerable technical difficulties in recovering the enamel matrix in decalcified sections; but, as a result of the pioneer work of Bodecker (1906), there has been, over the years, an increasing interest in this problem. Bodecker's early observations on organic elements in mature enamel, which, for many years, remained controversial,



FIGURES 1 to 5. Decalcified paraffin sections from jaws and teeth of rhesus monkey photographed at 100 times magnification and comparing alveolar bone (FIGURE 1); secondary cellular cementum (FIGURE 2); primary acellular cementum (FIGURE 3); dentin (FIGURE 4); enamel (FIGURE 5). Note the relatively wide zone of pale (acidophilic) uncalcified predentin.

FIGURES 6 to 9. Similar sections, photographed at 500 times magnification. Note the similarity in the size and shape of the osteocytes of alveolar bone (FIGURE 6) and the cementocytes of cellular cementum (FIGURE 7). In contrast observe the minute dimensions of the tubules of the dentin (FIGURE 8) and prism sheaths of the enamel (FIGURE 9).



FIGURES 10 to 15. Decalcified sections of mature human enamel. Note that the appearance of the Schreger's bands (FIGURE 10), incremental lines of Retzius (FIGURE 11), and tufts (FIGURE 12) can be explained by the developmental pattern of the organic framework of enamel. In contrast observe the irregularity of the so-called enamel lamella (FIGURE 13) suggesting independence of normal enamel formation. Compare the appearance of decalcified enamel in ordinary paraffin section magnified 1000 times under the ordinary microscope (FIGURE 14) with the significant details in ultrathin shadowcast section magnified 20,000 times under the electron microscope (FIGURE 15).

have since been confirmed by a number of other investigators and thus have brought the enamel somewhat closer into the family of hard tissues than was originally conceived. From our own participation in this research during recent years (Sognnaes, 1948-1950), FIGURES 10 to 15 summarize the morphological appearance of decalcified enamel from adult human teeth. The demonstration in *decalcified sections of mature enamel* of the Schreger's bands (FIGURE 10), Retzius lines (FIGURE 11), tufts (FIGURE 12), *etc.* (structural characteristics which, long ago, were recognized in ground sections) would now seem to justify the generalization that the histomorphology of the enamel can be largely attributed to the pattern of its organic framework.

In a category by themselves are the heavy bands of organic matter known as enamel lamellae (FIGURE 13). Their relative bulkiness, irregularity, and independence of normal developmental patterns suggest that these controversial structures (Bodecker *et al.*, 1951) may represent artificial separations or cracks between the enamel prisms, secondarily filled with organic matter which possibly originates, in part at least, from saliva, and may well serve some crude "reparative" function.

Of greater significance to our understanding of normal enamel is the recent finding that a delicate submicroscopic fibrillar framework, which is not observable within the resolving power of the ordinary microscope (FIGURE 14), can be demonstrated with the electron microscope (Scott, Ussing, Sognnaes, and Wyckoff, 1952) and permeates the core of the prisms as well as the interprismatic regions (FIGURE 15). This newest illustration of the wide distribution of organic elements in the enamel will be described in detail in the report to follow by Scott (1955), but it is included here so that it may be directly compared with the optical microphotographs.

For comparative purposes and for the sake of brevity, the developmental and functional characteristics of the four mineralized tissues are summarized in TABLES 2 and 3, respectively, which contain self-explanatory and generally accepted information with the exception of four items which require additional comment:

(1) With reference to TABLE 2, it should be mentioned that a third mode of

TABLE 2

PRINCIPAL DEVELOPMENTAL CHARACTERISTICS OF THE MINERALIZED TISSUES

Structure	Embryonic origin	Developmental mechanism	Uncalcified precursor	Appositional pattern	Structural type
Bone	Mesenchymal	Endochondral, intramembranous	Osteoid	Lamellae	Spongy, compact
Cementum	Mesenchymal	Intramembranous	Cementoid	Lamellae	Cellular, acellular
Dentin	Mesenchymal	Intramenbranous	Predentin	Incremental lines of Ebner	Tubular
Enamel	Epithelial	Secretory	Pre-enamel	Incremental lines of Retzius	Prismatic

TABLE 3

ESSENTIAL VASCULAR, CELLULAR, AND FLUID ENVIRONMENT OF THE MINERALIZED TISSUES

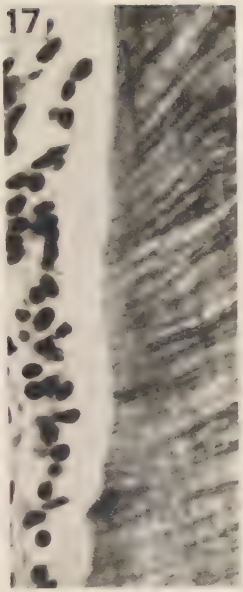
Structure	Internal vascular canals	Internal cellular spaces	Internal cellular elements	Adjacent cellular elements	Adjacent tissue fluid environment
Bone	Haversian, Volkmann's canals	Lacunae, canaliculi	Osteocytes	Fibroblasts Osteoblasts Osteoclasts	Connective tissue fluid
Cementum	None	Lacunae, canaliculi	Cementocytes	Fibroblasts Cementoblasts ("Cementoclasts")*	Connective tissue fluid
Dentin	None	Dentinal tubules	Odontoblastic processes	Fibroblasts Odontoblasts ("Odontoclasts")*	Connective tissue fluid
Enamel	None	None	None	None	Saliva

* Normally present and physiologically significant during shedding of deciduous teeth.

osteogenesis, neither endochondral nor intramembranous in nature, has been recently proposed; namely, the bone formation in antlers, which Wislocki, Weatherford, and Singer (1947) have termed "fibrovesicular" or "chondroidal" bone formation. Without going into further detail at this point, it is of interest to observe that this type of ossification is accompanied by certain morphological and histochemical characteristics which appear to be intermediary between those observed in skeletal and dental hard tissues, respectively.

(2) With reference to TABLE 3, it is suggested, by analogy rather than by evidence, that the fibroblasts adjacent to the osteoblasts and cementoblasts may possibly play a similar role in the development of the fibrous framework of bone and cementum, as the pulpal fibroblasts (FIGURE 16) have been reported to do in case of dentin formation (Korff, 1906). This concept by no means rules out a significant organizing influence of the odontoblasts, cementoblasts, and osteoblasts in shaping the morphological pattern of the mesenchymal hard tissues and in the elaboration and calcification of the amorphous ground substance.* Experimental injury to the odontoblasts does not necessarily stop formation of the fibrous protein of the predentin, but appears to prevent the normal elaboration and calcification of the ground substance. Thus, in contrast to normal dentin, in which there is a sharp transition between the acidophilic collagenous framework of the predentin and the more basophilic, calcifiable dentin (FIGURE 17), we find, after injury to the odontoblasts, an irregular and widened predentin, as well as acidophilic, uncalcified, interglobular dentin spaces (FIGURE 18). It is interesting to note that the condition here produced is very similar to that reported by Mellanby (1929) and others, in vitamin D deficiency; but the extent to which such faulty calcifications are mediated by deficiencies in the calcium metabolism alone or by failures in the elaboration of a calcifiable ground substance has not been fully established.

* It should be noted that the German word "*Grundsubstanz*", in Europe, has been applied to the collagen fibers of bone and dentin (Korff, 1906). In the present discussion, the English term "*ground substance*" will be applied to the amorphous part, and the term "*fibrous framework*" to the structurally oriented part of the organic matrix of the mineralized tissues.



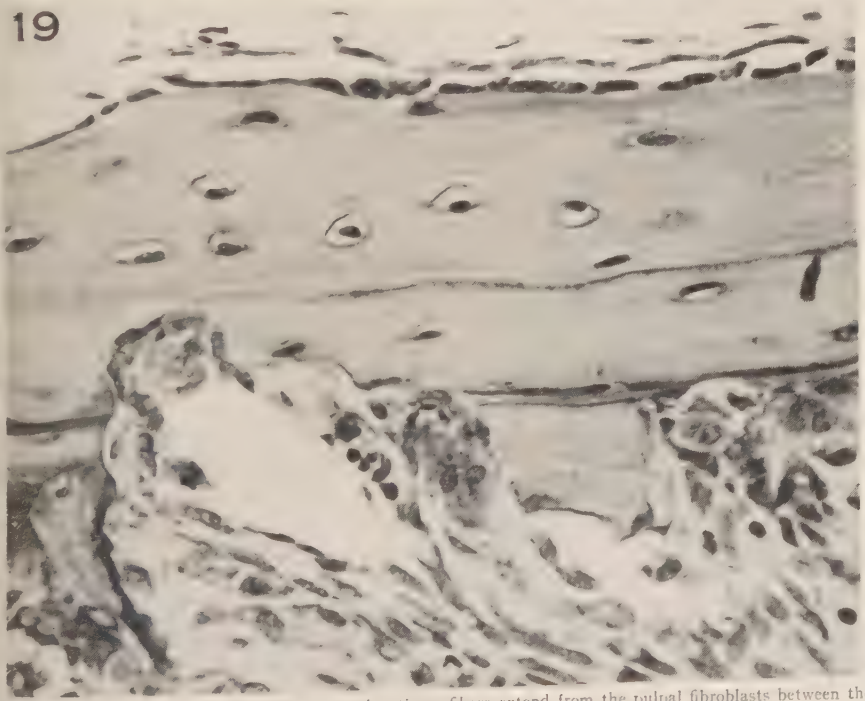


FIGURE 16. The so-called Korff's connective tissue fibers extend from the pulpal fibroblasts between the odontoblasts and spread out within the predentin zone (from original drawing by Korff, 1906)

FIGURE 17. Decalcified section of normal dentin shows from left to right: odontoblasts, wide pale (acidophilic) predentin zone and darker (more basophilic) calcifiable dentin

FIGURE 18. Decalcified section of abnormal dentin caused by experimental injury to odontoblasts and resulting in failure in the elaboration of the ground substance, a widened irregular zone of predentin and pale (acidophilic) uncalcifiable interglobular dentin spaces.

FIGURE 19. Decalcified section of alveolar bone undergoing circular remodelling. Note osteoblastic apposition above and osteoclastic resorption below, with one giant cell apparently situated within a blood vessel extending into Howship's lacuna.

(3) While the dental hard tissues are not normally surrounded by osteoclasts, there is a significant parallel to bone resorption during the physiological shedding of the deciduous teeth. In children between 6 and 13 years of age, one deciduous tooth after the other undergoes resorption in the presence of Howship's lacunae and giant cells which, for all practical purposes, appear like osteoclasts. In this condition, resorption not only affects the bonelike cellular cementum, but progresses into the acellular cementum and dentin, and occasionally into the enamel, until the tooth has lost its support and exfoliates. This is a matter of significance, because the latter hard tissues have no internal antecedent cells which could possibly contribute to the development of the osteoclast-like giant cells, indicating that these cells must originate from the adjacent soft tissue environment. In a study on the shedding of deer antlers, Wislocki and Waldo (1953) have similarly pointed to the significance of the extremely vascular fibrocellular connective tissue adjacent to the resorbing antlers, stressing active growth rather than death as the inducement for resorption. With reference to primate bone, an unusual microphotograph from the alveolar bone of a normal young rhesus monkey is shown in FIGURE 19, in which a giant cell appears to be within the wall of a blood vessel that is not only adjacent to the bone but actually situated in a typical Howship's lacuna. One can only speculate as to whether this cell is a large monocyte, a megakaryocyte, or an osteoclast in the making (Hancox, 1949). Suffice it to say, vascularity and giant cells appear to go hand in hand, and the acellular and avascular dental hard tissues do not appear to undergo lacunar resorption unless granulation tissue or other richly vascularized structures are nearby. It is far from clear, however, wherein lies the protective mechanism which normally appears to spare the teeth from resorption. It is particularly difficult to explain in the case of the bonelike cementum. Normally, this layer remains intact, whereas the adjacent alveolar bone is subject to physiological remodeling, in adaptation to growth and function; to therapeutic reconstruction in orthodontic tooth movement; and to pathological resorption in response to changes in the calcium metabolism, such as accompanies hyperparathyroidism (Albright and Reifenstein, 1948).

(4) With regard to enamel (TABLE 2), it is generally accepted that the primordium of the enamel prisms is elaborated and secreted by the ameloblasts, which, prior to this function, elongate, reverse their polarity, change the position of their Golgi apparatus, and exhibit cytoplasmic granules in the distal portion facing the dentin (Beams & King, 1933). Furthermore, it is suggested (TABLE 3) that the relationship of saliva to the enamel may be considered a parallel to the relationship of extracellular connective tissue fluid versus the mesenchymal hard tissues. Evidence for this concept of saliva and enamel as a liquid-solid system of potential significance in the physiology and pathology of the teeth will be given later under the discussion of form and function.

(2) *Histochemical Reactions*

The present discussion will be concerned mainly with the histochemical reactions which appear most pertinent to an understanding of the elaboration, nature, and calcification of the organic matrices of the mineralized tissues.

The histochemistry of cartilage and endochondral bone formation was reviewed a few years ago by Follis and Berthrong (1949) and has since received considerable attention in the annual proceedings of the Macy Foundation's Conferences on Metabolic Interrelations (1949-1952). While the behavior of cartilage, however, is of singular significance in the growth and pathology of the long bones (Wilkins, 1955), it is believed that the calcification process and behavior of hard tissues *per se* may be profitably observed in the flat bones of the skull, in the jaws, and in the cementum, dentin, and enamel of the teeth, since all of these hard structures are initiated, formed, and mineralized without cartilaginous precursors.

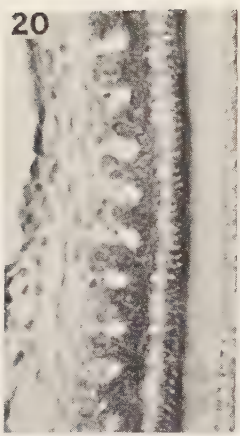
(1) *Enzyme reactions.* Alkaline phosphatase and its relation to bone biology has been recently reviewed by Majno and Rouiller (1951), who cite 100 of the numerous references to this subject. Mention will be made here concerning the results obtained with the recent modifications of the Gomori (1941) technique by Lorch (1946), and by Greep, Fischer, and Morse (1948). These two independently developed procedures make it possible to eliminate the otherwise confusing presence of calcium phosphate from the sections of mineralized tissues, so that the alkaline phosphatase reaction can be observed in thin decalcified paraffin sections of hard and soft tissues together.

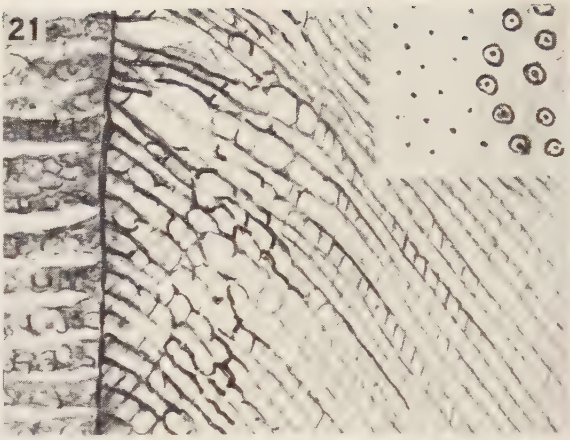
Using this technique, Greep, Fischer, and Morse have confirmed the wide distribution of alkaline phosphatase in the odontogenic and osteogenic cells. Using both the standard Gomori technique and the modified Greep-Fischer-Morse procedure, Wislocki and Sognnaes (1950) showed the presence of alkaline phosphatase both prior to and during the calcification period in the odontogenic cells of the enamel organ (FIGURE 20) and dental papilla. In addition, they observed a slight reaction in the region between the calcifying enamel prisms and in the predentin and dentinal tubules (FIGURE 21).

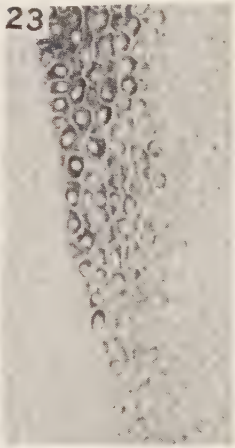
Bevelander and Johnson (1950) applied the Lorch method to developing and calcifying intramembranous bone of pig embryos, and observed that alkaline phosphatase could be revealed in the osteogenic cells prior to calcification of the bone and that the enzyme reaction, to a lesser degree, was present in the young calcifying matrix.

According to the work of Bradfield (1949), phosphatase activity appears to be related to elaboration of fibrous protein. In keeping with this concept, we have observed in a recent study (Sognnaes and Lustig, 1955), that the epithelial cells which form the hornifying "teeth" of the lamprey (FIGURES 22, 23, 24) in which no calcification takes place, react for alkaline phosphatase during elaboration of the fibrous elements fully as strongly as do the cells responsible for enamel and dentin formation in higher animals.

Majno and Rouiller (1951) applied the method of Lorch to bone, and reported that the enzyme reaction not only was present in the osteoblasts but in the osteoclasts as well. On that basis, they suggest that the enzyme is involved not only in bone apposition, through hydrolysis and deposition of PO_4, but also in bone resorption, through esterification, and withdrawal of PO_4. In unpublished work (Sognnaes and Rothblatt, 1954), we have made similar observations of the enzyme in osteoclasts of the alveolar bone, made to resorb experimentally







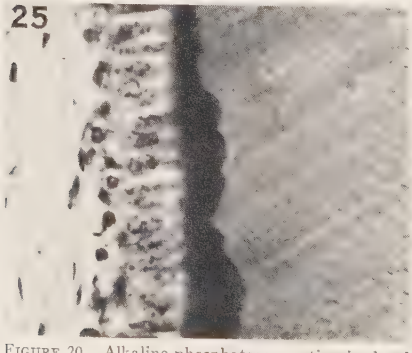


FIGURE 20. Alkaline phosphatase reaction in developing rat incisor; from left to right, stellate reticulum, stratum intermedium, ameloblastic layer, and young enamel matrix. Decalcified and reactivated according to the procedures of Greep, Fischer, and Morse (1948), giving negative control section.

FIGURE 21. Higher magnification of enamel from similar section to FIGURE 20; alkaline phosphatase activity is concentrated between calcifying enamel prisms and their cross striations. Insert illustrates enzyme reaction within dentinal tubules of young dentin (upper right corner). Modified after Wislocki and Sognnaes, 1950).

FIGURES 22 to 24. Germinal groove of horny "teeth" of the lamprey. Note that, despite the absence of calcification, there is an intense alkaline phosphatase reaction in the nuclei of the epithelial cells (FIGURE 22) associated with elaboration of sulfhydryl positive granules within the epithelial cytoplasm (FIGURE 23) and later throughout the cornifying epithelium (FIGURE 24).

FIGURE 25. Developing enamel of molar from rhesus monkey showing cytoplasmic granules in ameloblasts and transition from pre-enamel to young calcifying enamel matrix, which stains similar to cornifying epidermis, Masson stain.

FIGURE 26. Pre-enamel matrix from rat incisor stained with Chevremont and Frederic's Prussian blue method for sulfhydryl groups (modified after Wislocki and Sognnaes, 1950).

by functional stress according to the method of Waldo and Rothblatt (1954). Majno and Rouiller suggest that the alkaline phosphatase reaction distinguishes the osteoclasts from other giant cells such as the Langhans cells, which, according to Gomori (1943), do not contain the enzyme, and the megakaryocytes which, according to Wislocki and Dempsey (1946), also react negatively.

On the basis of histochemical observations, it would appear that alkaline phosphatase is demonstrable in connection with the whole life cycle of the mineralized tissues, *i.e.*, matrix formation, calcification, as well as resorption.

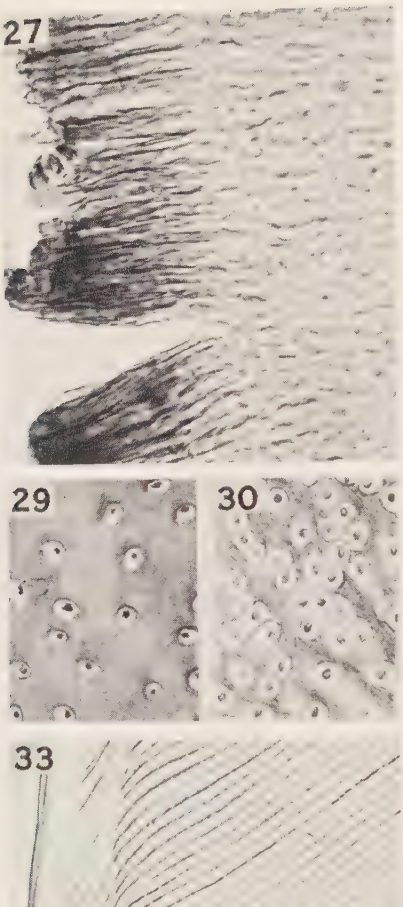
Histochemical demonstration of other enzymes has been very limited compared to alkaline phosphatase. Majno and Rouiller feel uncertain of the demonstration of acid phosphatase in osseous tissues. Follis and Berthrong were dissatisfied with the available methods for demonstration of succinic and citric acid dehydrogenases in various regions of cartilage, in relation to bone formation and calcification. In free hand slices of cartilage and bone from rats and guinea pigs, they observed reactions attributed to the presence of cytochrome oxidase in the nuclei of the osteoblasts adjacent to young bone spicules. However, they did not wish to interpret the significance of this enzyme in its relation to osteoid formation and bone salt deposition.

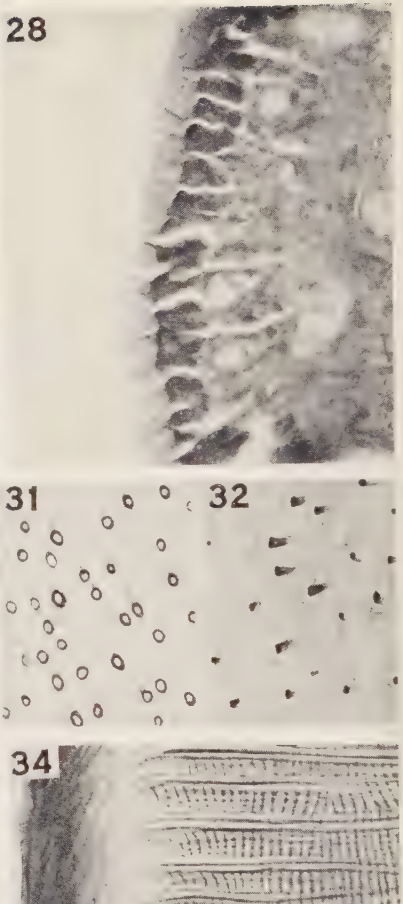
Finally, it is of interest that Pincus (1953), by biochemical means, has recently reported the presence of cytochrome *c* and succinic oxidase systems in human dentin and pulp.

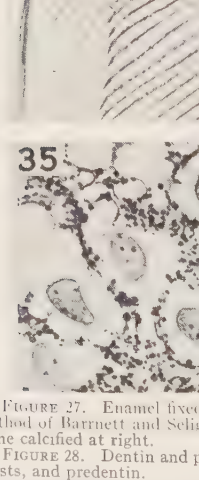
(2) *Sulfhydryl and disulfide reactions.* Following Bodecker's early work on the histological recovery of the organic framework of calcified enamel, Rosebury (1930) made the first extensive chemical study of this organic matrix. From mature enamel, he recovered 0.4 per cent of an alkali- and acid-resistant residue giving various chemical reactions suggestive of keratin. More recent chemical work on the isolation and composition of enamel keratin is reviewed elsewhere in this monograph (Stack, 1955). Histologically, it has been recognized for some time that the enamel matrix shares certain staining reactions with cornifying epidermis (Chase, 1929). For example, whereas the standard Masson triacid method stains dentin green, like bone and other connective tissues, the enamel stains reddish, and is thus more comparable to epithelial structures. Furthermore, a distinction can usually be made between the deeper staining pre-enamel matrix and the calcifying portion (FIGURE 25). These two regions have been found to correspond to a transition from a homogeneous to a fibrillar organic framework, according to recent observations with the electron microscope (Sognnaes, Scott, Ussing, and Wyckoff, 1952).

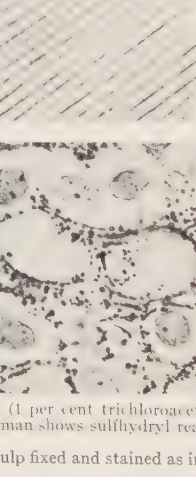
With more specific histochemical methods, Wislocki and Sognnaes (1950) have shown the presence of sulfhydryl reactions in the young enamel matrix (FIGURE 26), using among other procedures, the Prussian blue reaction of Chevremont and Frederic (1943). Recently we have used the new methods of Barrnett and Seligman (1952) and Barrnett (1953), with which SH groups and SS bonds can be more specifically distinguished, with the following preliminary results (Sognnaes, unpublished data):

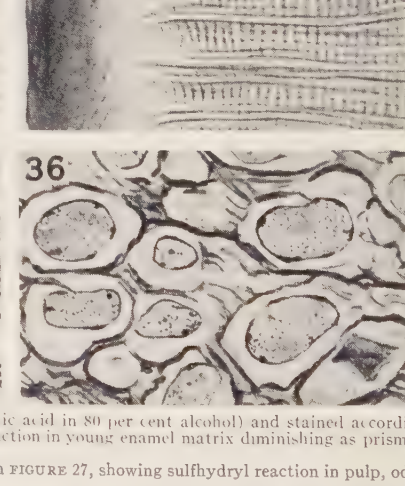
A sulfhydryl positive reaction has been observed not only in the ameloblasts and pre-enamel matrix (FIGURE 27) but to a lesser degree in the pulp, the odon-

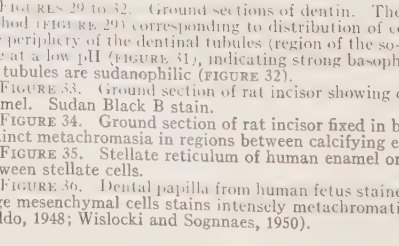












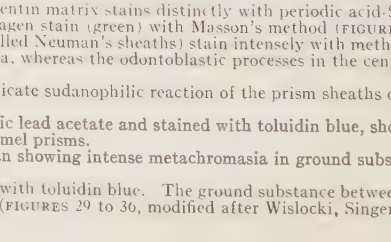


FIGURE 27. Enamel fixed (1 per cent trichloroacetic acid in 80 per cent alcohol) and stained according to method of Barrnett and Seligman shows sulfhydryl reaction in young enamel matrix diminishing as prisms, become calcified at right.

FIGURE 28. Dentin and pulp fixed and stained as in FIGURE 27, showing sulfhydryl reaction in pulp, odontoblasts, and predentin.

FIGURES 29 to 32. Ground sections of dentin. The dentin matrix stains distinctly with periodic acid-Schiff method (FIGURE 29) corresponding to distribution of collagen stain (green) with Masson's method (FIGURE 30). The periphery of the dentinal tubules (region of the so-called Neuman's sheaths) stain intensely with methylene blue at a low pH (FIGURE 31), indicating strong basophilia, whereas the odontoblastic processes in the center of the tubules are sudanophilic (FIGURE 32).

FIGURE 33. Ground section of rat incisor showing delicate sudanophilic reaction of the prism sheaths of the enamel. Sudan Black B stain.

FIGURE 34. Ground section of rat incisor fixed in basic lead acetate and stained with toluidin blue, showing distinct metachromasia in regions between calcifying enamel prisms.

FIGURE 35. Stellate reticulum of human enamel organ showing intense metachromasia in ground substance between stellate cells.

FIGURE 36. Dental papilla from human fetus stained with toluidin blue. The ground substance between the large mesenchymal cells stains intensely metachromatic (FIGURES 29 to 36, modified after Wislocki, Singer, and Waldo, 1948; Wislocki and Sognnaes, 1950).

toblasts, and the predentin (FIGURE 28). As the dentin calcifies, this reaction disappears without being replaced by a perceptible disulfide bond reaction. In enamel, on the other hand, the reaction is replaced, in part, by a reaction for disulfide bonds, as the enamel matures and calcifies. The relative weakness of this SS reaction in enamel compared to that in other cornified tissues is in keeping with the fact that the cystine content of enamel is very low, namely about 1 per cent (see Stack, 1955). In a comparative study of SH and SS reactions in cornified structures from various animals, we have so far observed the strongest SS reaction in rhinoceros horn, whereas human enamel is at the lower end of the scale (Barrnett, Sognnaes, and Pettengill, 1955). The relatively greater intensity of the sulfhydryl reaction in the pre-enamel matrix (compared to the subsequent SS reaction), and the simultaneous presence of the SH reaction in predentin and the odontogenic cells, are believed to be due to some other function of the SH groups, possibly associated with an oxidizing enzyme system related either to formation of the matrix or to its maintenance in an uncalcified state. It is known that sulfhydryl groups are important in oxidizing enzyme systems concerned with both fat, carbohydrate, and amino acid metabolism; but, with the exception of its relation to keratinization, relatively little progress is reported to have been made in our understanding of the role of sulfhydryl groups during the last 20 years (Barron, 1951).

(3) *Polysaccharide reactions.* Earlier literature on the presence of *glycogen* in osteogenesis has been reviewed by Follis and Berthrong (1949), who also discussed the possible significance of glycogen in calcification, both with regard to the phosphatase and phosphorylase mechanism. In endochondral calcification, they and others have demonstrated that the glycogen reaction increases in the cytoplasm of the cartilage cells as cartilage matures. Once mineral deposition begins, however, little or no glycogen is present in the adjacent cartilage cells.

Engel (1948) has tabulated earlier observations and added his own on the presence of glycogen in the dental tissues. From this and other recent references to the subject (Wislocki and Sognnaes, 1950), there appears to be general agreement that glycogen is present in large amounts in the oral epithelium, dental lamina, outer enamel epithelium, and stellate reticulum of fetal teeth. Furthermore, Wislocki, Singer, and Waldo (1948) have observed a perceptible reaction for glycogen in the stratum intermedium, ameloblasts, odontoblasts, and dental papilla of a human fetus of 130 mm. crown-rump length. On the other hand, glycogen could not be demonstrated in adult teeth of man or rhesus monkeys, either in the pulp, dentin, or enamel, by histochemical means. However, in a recent study, Egyedi (1953) claims to have demonstrated chemically the presence of glycogen in the insoluble portion of the organic matrix of enamel and, to a lesser extent, in dentin, but this cannot be evaluated until a more detailed report is available.

In 1948, Wislocki, Singer, and Waldo recorded the observation of a periodic acid-Schiff positive reaction (McManus, 1946) in the interstitial matrix of undecalcified ground sections of dentin from teeth of man and rhesus monkeys (FIGURE 29), corresponding to regions which also take routine stains for collagen

(FIGURE 30). The same year, Engel reported the presence of a *carbohydrate-protein complex* in the dentin and enamel matrix of rats' teeth, fixed by freeze-drying and stained by the Hotchkiss (1948) method. He also observed glycoprotein granules in the cytoplasm of the odontoblasts and ameloblasts. This same reaction, attributed to a polysaccharide component of the ground substance, was similarly demonstrated in undecalcified sections of human trabecular bone by Levine, Rubin, Follis, and Howard (1949). Similarly, Bevelander and Johnson (1950), using freeze-drying fixation and the Hotchkiss procedure, found a distinctly positive periodic acid-Schiff reaction in undecalcified paraffin sections of the calcifying spicules of intramembranous bones from the jaws of pig embryos. By the same method, Heller-Steinberg (1951) made similar observations in young rat bones and also described Schiff-positive cytoplasmic granules in the osteoblasts.

In apparent conflict with these findings is the observation of Rogers (1949), who also used the Hotchkiss technique, but reported essentially negative reactions in both bone and dentin matrix, whether prepared as undecalcified or decalcified sections. At the moment, his findings cannot be evaluated since he used certain modifications in the technique which were not fully described in his brief preliminary report.

With this reservation, it would appear that histochemical observations made to date favor the interpretation that a periodic acid-Schiff positive polysaccharide component is present in the interstitial matrix of the calcifying and calcified tissues described above.

In addition to the periodic acid-Schiff method of McManus and Hotchkiss, which has been most widely used for histochemical observations on polysaccharides, various hard tissues have been examined for the presence of metachromasia following toluidine blue staining, and basophilia following methylene blue staining at various lower pH levels. At neutral pH, ground sections stain only faintly or fail to stain in regions of fully mineralized matrix of bone (Levine, Rubin, Follis, and Howard, 1949), dentin (Wislocki, Singer, and Waldo, 1948), enamel (Wislocki and Sognnaes, 1950), and cementum (Lorber, 1951). In the periphery of the dentinal tubules, however, Wislocki, Singer, and Waldo (1948) have demonstrated a substance which is not only metachromatic when stained with toluidine blue, but also strongly basophilic when stained with methylene blue at a pH as low as 2 to 3 (FIGURE 31). This reaction surrounds the odontoblastic processes, which are not strongly basophilic, but instead exhibit a sudanophilic reaction (FIGURE 32). A less distinct sudanophilic reaction has been observed in the prism sheaths of the enamel (FIGURE 33). Furthermore, Wislocki and Sognnaes (1950) have described a strong metachromatic reaction of calcifying enamel in the interprismatic region and cross striations of the prisms (FIGURE 34). Finally, Lorber (1951) has noted similar reactions around the Sharpey's fibers in cementum. These are all regions which either remain as relatively uncalcified organic pathways or in which calcification is late to occur.

As to the origin of the metachromatic reaction in the teeth, it should be noted that during formation, metachromasia is very marked in the stellate reticulum of the enamel organ (FIGURE 35) adjacent to the enamel, in the dental papilla (FIGURE 36) adjacent to the dentin, and in the dental sac (Wislocki and Sogn-

naes, 1950) adjacent to the primary cementum. Moreover, it is noteworthy that a distinct metachromatic and basophilic reaction has been observed in the ground substance of the stellate cells in the germinal zone of deer antlers, and in the walls of the cellular lacunae and their ramifications in the precosseous columns (Wislocki, Weatherford, and Singer, 1947).

The fully calcified matrix of the mineralized tissues is difficult to stain unless the histochemical method calls for an acidic fixative or acidic reagents, such as the periodic acid-Schiff procedure.* The fixative of choice for demonstration of metachromasia is basic lead acetate, after which the fully mineralized matrix of bone is poorly stained, that of calcified enamel completely unstained (Wislocki and Sognnaes, 1950). Variable degrees of metachromasia observed following brief treatment with dilute nitric acid may suggest unmasking of the ground substance as suggested by Levine, Rubin, Follis, and Howard (1949), but Heller-Steinberg (1951) has demonstrated that the metachromasia of bone is greatly variable depending upon the pH of solutions employed, the mode of fixation, dehydration, and staining time.

This finding is in keeping with our own experience. In unpublished work (Sognnaes and Rothblatt, 1954), we have noted, furthermore, that the calcified matrix of bone, although periodic acid-Schiff positive, is not strongly basophilic when stained at lower pH levels with the method of Wislocki, Bunting, and Dempsey (1947). According to these authors, the histochemical demonstration of a strong metachromatic and basophilic reaction together is considered indicative of a substance identical with or closely allied to the sulfated acid mucopolysaccharide of cartilage and mucin.

Sylven (1947) has reported that the normal process of endochondral ossification is characterized by a rapid disappearance of metachromatic chondroitin sulfate from the cartilaginous matrix, concurrently with a great increase in alkaline phosphatase in adjacent cells. It is of interest, in this connection, that reactions attributed both to an acid mucopolysaccharide and to phosphatase occur coincidentally in the pulp of growing teeth (Wislocki and Sognnaes, 1950). In rats' incisors the growing roots are the sites of the greatest concentration of these substances, whereas, towards the crowns, both diminish. An association of these two substances exists also in the stellate reticulum of the enamel organ, in the enamel, and in the tubules of the predentin. Conditions in growing, calcifying teeth, therefore, suggest that acid mucopolysaccharides and alkaline phosphatase are not, necessarily, inversely related to one another in amounts, or mutually exclusive. The disappearance of metachromasia in fully mineralized areas, according to Sylven and others, is due to depolymerization or dis-

*Lillie (1950) has recently evaluated the validity of the polysaccharide reaction in great detail. Regarding the periodic acid-Schiff method, he lists a variety of substances and anatomical elements giving this reaction, among others: certain zymogen granules, probably albuminous; various pigments, hexose polysaccharides (glycogen, starch, and cellulose), the glycoprotein amyloid, protein, collagen, gelatin, and reticulin, sometimes coagulated plasma, serous exudates and fibrin, mucoid polysaccharides containing neuraminic and chondroitin sulfuric acids, and, according to some investigators, also the hyalauronic acid mucins and various granules, pigments, and colloids of the endocrine organs.

Furthermore, Lillie discusses the effect of various salt mixtures upon metachromasia and concludes that the production of a metachromatic color with toluidine blue does not necessarily prove that all other substances producing a metachromatic stain are polysaccharide sulphuric acid ester complexes, even though cartilage matrix and some of the mucins readily give this reaction and contain complexes of that nature.

The earlier interpretations of Lison (1936) and Sylven (1947), who considered metachromasia indicative of chondroitin sulphate, are therefore not consistent with the more recent observations made by Lillie (1950), as well as by Michaelis (1947), by Wislocki, Bunting, and Dempsey (1947), by Bunting (1950), and by Heller-Steinberg (1951).

appearance. However, as already pointed out above, there is still some doubt as to the validity of the metachromasia within the matrix. Furthermore, it seems probable that the amorphous substance, in the fully calcified regions, represents only a thin submicroscopic coating of the fibrils and crystals—possibly serving as a bond between the two a layer too thin to be observed readily with the optical microscope, whether stained or not.

While a final interpretation of the histochemical polysaccharide reactions cannot be made at this time, it may be significant that a consistently strong metachromasia is shared by cartilage, Wharton's jelly, nucleus pulposus, and cornea, structures which, like cementum, dentin, and enamel, are either avascular, acellular, or both. With reference to the work of Duran-Reynals (1942), Bunting (1950) suggests that the substance responsible for the metachromasia may contribute to the distribution of extracellular substances within tissues that are devoid of an intrinsic blood supply, such as cartilage. This suggestion would seem even more applicable to the dental hard tissues, and evidence will be presented below to the effect that the influx of phosphorus *within* the enamel prisms is remarkably high at the time when the metachromatic and alkaline phosphatase reactions *between* the prisms are most conspicuous.

(4) *Other organic components.* Hutton and Nuckolls (1951) have observed both superficial and internal staining of human enamel exposed to ninhydrin, a reagent which indicates the presence of *peptides.* They used unerupted human teeth, which may not have been fully mineralized. However, Stack (1952, 1955), using the same type of reagent, has determined that both unerupted and erupted human enamel contain peptide material accounting for one fourth of the total organic matter. The origin and physiological significance of this organic fraction would seem to warrant a great deal of attention. With regard to origin, the saliva may be ruled out as far as the unerupted teeth are concerned. Internally the enamel tufts and spindles may be suggested as the most logical pathways by which low molecular polypeptides and amino acids may be transported from the pulp into the enamel of unerupted as well as erupted teeth.

It is regrettable that there is no histochemical technique whereby the relative distribution of citric acid can be determined within the mineralized tissues, which contain about 70 per cent of the total amount present in the body (Dickens, 1941). Thunberg (1941) found 0.5 per cent citric acid in whole teeth and 0.08 to 0.35 per cent in the shell of eggs. Zipkin and Piez (1950) have recently found that there is about 0.9 per cent citric acid in dentin and 0.1 per cent in enamel, which, according to Stack and Williams (1952) accounts for about one sixth of the total organic matter present in whole enamel. It would be of considerable interest to learn the relative distribution of citric acid in predentin and calcified dentin. Such histochemical information might shed light on the part which this prominent organic component plays in normal (Carter, 1951) and pathological calcification, particularly with regard to vitamin D deficiency (Steenbock and Bellin, 1953; Nicolaysen and Eeg-Larsen, 1953).

(5) *Mineral deposition.* Microscopic visualization and identification of mineral deposition in tissues were made years ago by microincineration of microscopic sections. Hampp (1940) applied this procedure to developing teeth,

TABLE 4
MAIN COMPONENTS OF THE MINERALIZED TISSUES

Main components	Bone	Cementum	Dentin	Enamel
Inorganic crystals	Apatite	Apatite	Apatite	Apatite
Ground substance	Polysaccharide	Polysaccharide	Polysaccharide	Polysaccharide
Fibrous framework	Collagen	Collagen	Collagen	"Keratin"
Cell components	Osteocytes	Cementocytes	Cell processes	None
Blood vessels	Present	None	None	None

but little further has been done. As modern means to the same end, the widening scope of histochemistry may be illustrated by the elegant technique of historadiography, recently applied to bone by Amprino and Engström (1952) and the now widely used radioautographic procedures, the fruitful use of which is illustrated elsewhere in this monograph (Leblond, Bélanger, and Greulich, 1955) and will not be discussed at this point.

(6) *Organic-inorganic linkage.* From the foregoing discussion, it is evident that, in order to find common grounds for comparison of the four mineralized tissues, one must refer to the components which, in the beginning, were defined as the fourth structural order (TABLE 1). On this basis, we may tentatively chart the main components of the hard tissues as shown in TABLE 4. The inorganic crystals will be discussed elsewhere in this monograph by Hendricks (1955) and Trautz (1955). It is of interest, from a morphological standpoint, that the crystals of enamel have recently been viewed and measured directly in sections examined under the electron microscope and appear to be of considerably larger dimension than those of the mesenchymal hard tissues (Scott, Ussing, Sognnaes, and Wyckoff, 1952; Sognnaes, Scott, Ussing, and Wyckoff, 1952). The exact nature and relative quantity of ground substance cannot be discussed on the basis of histochemical findings, except to indicate that polysaccharide reactions suggesting the presence of a mucoprotein is found in all mineralized tissues. In the fibrous framework, there are not only obvious quantitative variations but, in addition, distinct qualitative differences between enamel and the other hard tissues. The fibrous protein of the enamel has been classified as a keratin or, more specifically, as a eukeratin (Block, Horwitt, and Bolling, 1949). Compared to hair and nail keratin, however, it seems less oriented, more soluble, and, according to Block (1951), relatively high in phenylalanine while very low in cystine. It is interesting, furthermore, that Hess, Lee, and Neidig (1953) and Stack (1953) have reported the presence of the amino acid hydroxyproline in the keratin of enamel. During a discussion of collagen that accompanied a recent Macy Conference on calcification, Stetten (1951) suggested the possibility that the hydroxyl groups of hydroxyproline may perhaps have a specific function as points of attachment for carboxyl groups of the polysaccharides in the ground substance of bone. This possibility was proposed as a pure conjecture, but it is mentioned here since it may pertain to all of the mineralized tissues, including enamel. The linkage between the inorganic crystals, ground substance, and fibrous framework cannot be elucidated by ordinary microscopy. Submicroscopic morphological observations on this question, as the problem

pertains to collagen and crystals of bone, will be discussed in this monograph by Robinson and Watson. Among known chemical ingredients, citric acid deserves attention as a conspicuous organic component shared by all of the mineralized tissues.

(3) *Form and Function*

From these data, it would appear that the development of the mineralized tissues, whether osseous or dental in nature, require several similar ingredients and, hence, must be basically controlled by comparable mechanisms of formation and calcification. There is particularly good evidence that developing teeth respond as sensitively as bone to various nutritional factors (Shaw, 1955; Sobel, 1955).

A distinction, however, must be made between the behavior of the various hard tissues once they are fully calcified and in function. The reconstruction of bone associated with functional adaptation and growth, and the dynamic participation of the bone trabeculae, as a readily available reserve supply of calcium (Aub, 1928–1929; Bauer, Aub, and Albright, 1929), are not shared by the dental hard tissues. Teeth, unlike bones, are formed and calcified into their final size and shape during the primary development, the deciduous teeth so as to be accommodated within the jaws of the child, the permanent teeth within the jaws of the adolescent and adult. Except for the shedding of the deciduous teeth, previously referred to, no one has ever demonstrated histological landmarks to indicate that any portion of the enamel, dentin, and cementum of fully formed teeth is *physiologically* removed by cellular resorption. Even if the teeth were drawn upon as a source of calcium, the dental hard tissues could contribute very little to the metabolic pool. Of the more than 1000 grams of calcium in the body of an adult man, the ratio of the total calcium content of bone, dentin, enamel, and cementum, respectively, is about 5000:25:10:1 (TABLE 5). In fact, there is no more calcium in the enamel of the whole human dentition than what has been shown to be withdrawn from the bone trabeculae of the pigeon during the egg-laying cycle (W. Bloom, M. A. Bloom, and McLean, 1941). Were not human teeth protected from such dynamic metabolic resorption, they would literally be "scarce as hen's teeth."

At the submicroscopic or chemical level of observation, no such sharp distinction can be made between osseous and dental hard tissues. Little is yet

TABLE 5

APPROXIMATE CALCIUM CONTENT OF BONES, TEETH, BLOOD AND SALIVA OF ADULT MAN

Sample	Calcium content (per cent)	Total calcium (grams)	Calcium ratios
Bone	20	1000	5000
Dentin	25	5	25
Enamel	35	2	10
Cementum	20	0.2	1
Blood	10 mgm. %	0.2	1
Saliva	5–10 mgm. %	(0.1 g./day)	—

known regarding the response of the dental hard tissues to changes in ionic concentrations of the tissue fluids such as accompanies acidosis and alkalosis (*cf.* W. Neuman and M. Neuman, 1953). But the significant concept of exchange which developed from the use of radioactive isotopes as biological tracers (Chievitz and Hevesy, 1935), soon brought the dental hard tissues closer into the orbit of mineral metabolism. The significance of this concept has recently been extensively reviewed in relation to both phosphorus metabolism (Armstrong, 1952), calcium metabolism (Nicolaysen, Eeg-Larsen, and Malm, 1953), and the mineral phase of bone in general (W. Neuman and M. Neuman, 1953). Further discussion of this problem is also covered elsewhere in this monograph by three of the most active contributors to the field (Armstrong, 1955; Hodge, 1955, and W. Neuman and Weikel, 1955).

With reference to the result of such recent radiotracer studies, it was suggested in a previous paragraph that saliva may serve as a tissue fluid to the enamel in a relationship comparable to that of extracellular connective tissue fluid to the mesenchymal hard structures (TABLE 3). The observations upon which this concept is based will now be described.

Following intravenous administration of radiophosphorus, it has been observed that there is an elevated radioactivity in the outer layer of the enamel which can be abolished by preventing the circulating radioactive saliva from reaching the enamel surface (Sognnaes and Volker, 1941). This original finding was made in cats and dogs, using low levels of cyclotron-made radiophosphorus. During the last few years, this observation has been confirmed and extended in more elaborate studies in the primate (rhesus monkeys) and employing much higher specific activities (Sognnaes and Shaw, 1952).

The general arrangement of these experiments is shown in FIGURE 37, which illustrates the three main experimental conditions employed: (1) radioisotope was allowed to reach the teeth both from the internal blood supply and the external saliva (control); (2) the intravenously injected isotope was allowed to reach the teeth through the internal blood supply while the enamel exposed in the mouth was capped by plastic tubes and surrounded by nonradioactive saliva; (3) the enamel was exposed to radioactivated saliva in order to observe the uptake and penetration of radioisotope from the external without internal administration of radioisotope.

Histochemical evidence of the radioisotope uptake was obtained by a radioautographic technique employing methylmethacrylate embedding whereby both hard and soft tissues could be sectioned together (Sognnaes, Shaw, Solomon, and Harvold, 1949), as illustrated in FIGURES 38 and 39. Findings so obtained were implemented by quantitative data on the radioisotope distribution in samples obtained by grinding with diamond stones and steel burs from various layers of enamel and dentin, respectively, as illustrated in FIGURE 40.

In control animals, following intravenous radiophosphorus injection, all hard tissues exhibit gradients in radioactivity increasing towards the regions in closest proximity to the adjacent fluid environment. One of the most recent experiments illustrating these gradients is shown in TABLE 6 (Sognnaes, Shaw and Bogoroch, 1955). Calcifying enamel, as already noted in the radioautographs

PROCEDURE FOR STUDYING RADIOISOTOPE UPTAKE AND PERMEABILITY OF DENTAL TISSUES

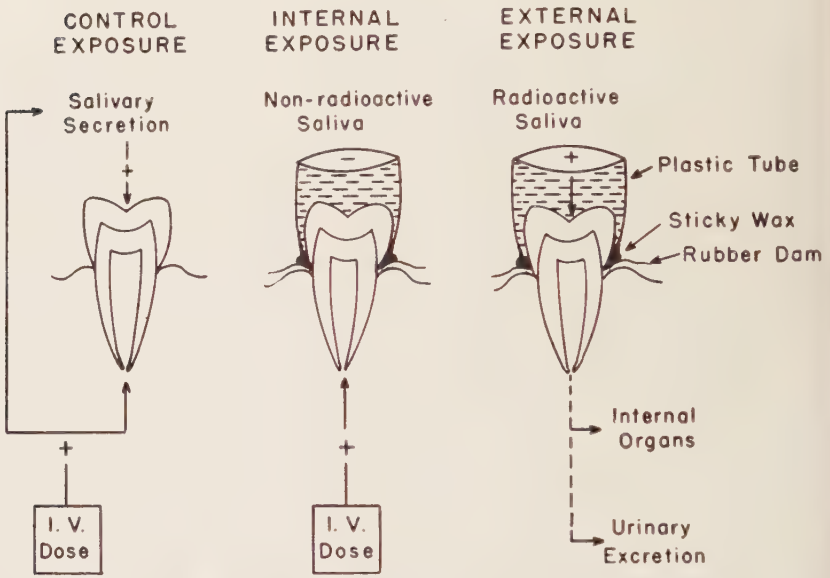


FIGURE 37. Diagram showing general experimental design for studying permeability and uptake of radioactive isotope in the teeth of rhesus monkeys

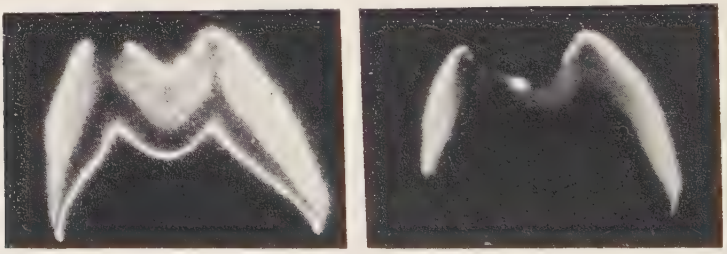


FIGURE 38. Radioautographs of plastic-embedded ground sections from unerupted molars of radiophosphorus-injected rhesus monkey. In section at right the dentin was removed from the section by a dental drill prior to radioautographic exposure (method of Sognnaes, Shaw, Solomon, and Harvold, 1950).

(FIGURE 39), takes up extremely high amounts of phosphorus throughout its thickness, as shown quantitatively in TABLE 6.

In erupted teeth, two gradients can be observed, the one decreasing from the internal to the external dentin, the other increasing from the internal to the external enamel. The first gradient can be experimentally eliminated by removing the pulpal blood supply; the second, by preventing the radioactive saliva from coming in contact with the enamel, as shown in TABLE 7. However, even in the absence of radioactive saliva, it appears from these and other experiments that a small amount of intravenously injected isotope reaches the in-

FIGURE 39. Above: X-ray photograph of maxilla of rhesus monkey comparable in age to that described in TABLE 6. Note that the crown of the teeth is fully formed and appears well advanced in calcification. Below: radioautographs of plastic-embedded sections from lower jaw of same animal as above. Note the radioactive gradients in the erupted teeth, and the uniformly high activity throughout the thickness of the enamel of the unerupted premolar (second from left).

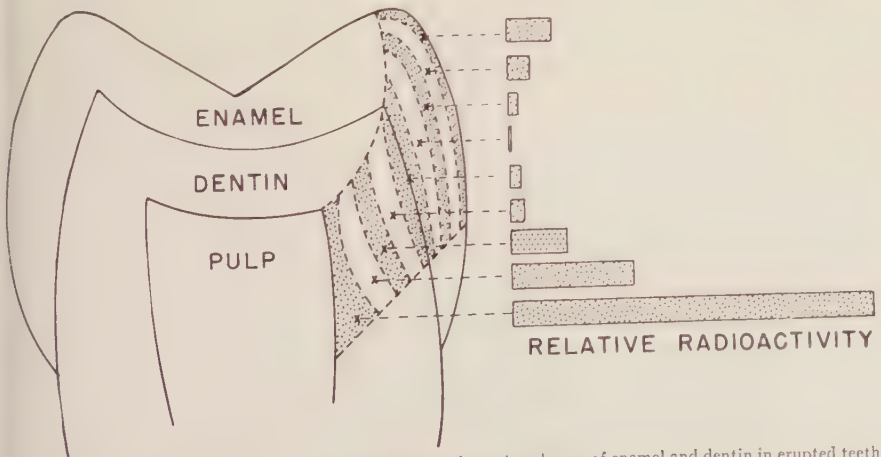


FIGURE 40. Relative distribution of radiophosphorus in various layers of enamel and dentin in erupted teeth of intravenously-injected rhesus monkeys (average for 11 control animals, according to data from Sognnaes and Shaw, 1952).

TABLE 6

RADIOPHOSPHORUS DISTRIBUTION IN OSSEOUS AND DENTAL HARD TISSUES OF THE RHESUS MONKEY 11 DAYS AFTER I. V. INJECTION OF 5 MC. P^{32}.

(From Sognnaes, Shaw, and Bogoroch, 1955)

Hard tissues examined	Adjacent environment	P^{32} gradients in the hard tissues*		
		1st layer†	2nd layer	3rd layer
Bone				
Humerus—prox. neck	Periosteum	2800	3300	3600
Humerus—shaft	Endosteum	1300	300	220
Mandible—alveolus	Periodontal membrane	2200	1700	1800
Cementum (root)				
M_1—cellular	Periodontal membrane	900	550	350‡
M_1—acellular	Periodontal membrane	540	190‡	160‡
Dentin (crown)				
P_1—calcifying	Dental papilla	1600	150	150
M_2—maturing	Pulp	530	110	80
M_1—functioning	Pulp	350	140	60
Enamel				
P_1—calcifying	Enamel organ	2300	2300	1600
M_2—maturing	Gum tissue	880	350	200
M_1—functioning	Saliva	20	3	2

* Per cent of injected dose × 10^7 per mgm. of air dried powder.
† Surface layer in direct contact with the adjacent environment listed above.
‡ These samples unavoidably included some of the underlying root dentin.

TABLE 7

EFFECT OF SALIVARY AND PULPAL ENVIRONMENT ON THE DISTRIBUTION OF P^{32} (COUNTS/MIN./MGM.) IN DENTIN AND ENAMEL OF RHESUS MONKEYS

Tissue area	Control	Salivary P^{32} absent	Pulpal P^{32} absent
Enamel			
External	58	2	49
Layer 2	17	7	10
Layer 3	20	11	4
Layer 4	26	3	24
Dentin			
Layer 1	48	46	3
Layer 2	105	94	0
Layer 3	467	385	0
Internal	490	498	0

ternal enamel. From these and other observations (Sognnaes, Shaw, Bogoroch, and Sweeney, 1952), it is concluded that the teeth are permeable to various ions in both directions, and that a certain turnover takes place in the enamel resulting mainly from the salivary secretion but, to a lesser degree, from the internal blood supply of the teeth, presumably by way of the dentinal tubules, dentino-enamel junction and, possibly, from the enamel spindles and tufts. A recent experiment (Fanning, Shaw, and Sognnaes, 1954) suggests that the salivary environment may be a significant factor during the final maturation period of the enamel as the teeth emerge into the mouth, and may indirectly influence the subsequent susceptibility of the teeth to dental caries.

On the basis of the various observations referred to above, the diagram shown

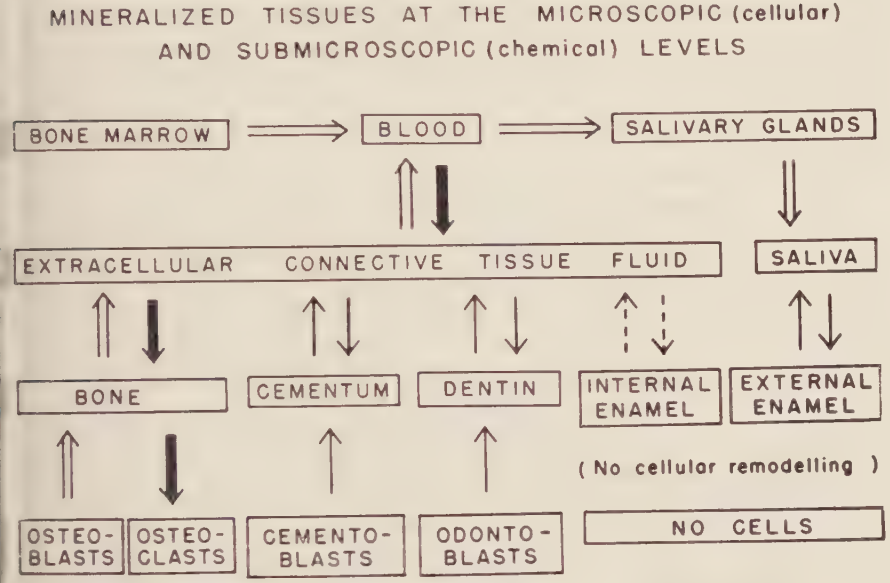


FIGURE 41. Diagram summarizing the interrelationships between the various mineralized tissues and their adjacent cellular and fluid environments

in FIGURE 41 has been prepared as a means of comparing the functional potentialities of the four mineralized tissues. These potentialities may be summarized as follows: whereas bone has a mechanism for continuous apposition and resorption accompanied by microscopically demonstrable changes within the hard tissue and its adjacent cells, the cementum and dentin are *normally* subject only to a one-way process of cellular activity, namely, secondary apposition in connection with masticatory function and wear. At the other end of the spectrum, the acellular and avascular enamel is completely protected from microscopically demonstrable resorption or apposition, but shares with the other mineralized tissues a turnover of inorganic components.

With respect to the turnover of the organic components, very little is known, as yet, about bone, and next to nothing about teeth. Schubert and Armstrong, (1948) made a preliminary study in which they observed a certain withdrawal of radiocarbon-labeled organic constituents from bones and teeth. However, in the case of the fully grown teeth (rat molars) no distinction was made between the organic and inorganic origin of the radioisotope.

Another intriguing observation pertinent to this matter is the recent report of Engel (1952) that the amount of mucoproteins in plasma almost tripled in 48 to 96 hours after parathyroid hormone injection, suggesting the possibility that at least some of this mucoprotein originated from the ground substance of bone.

In the case of osteoclastic resorption, it seems obvious that all organic and inorganic ingredients of the hard tissues are removed, calcium being the most

readily measured. However, much more exploration is necessary in order to learn to what extent the various organic and inorganic constituents of the hard tissues can be withdrawn, accrued, or turned over selectively without cellular remodeling, and what additional physiological significance can be attributed to the various components of the mineralized tissues in the maintenance of general, skeletal, and dental health.

Summary

(1) The microstructural organization of the four mineralized tissues is comparable insofar as they all contain (a) a *fibrous framework*, (b) an *amorphous ground-substance*, and (c) *inorganic crystals*, deposited in that order, whereas the vascular and cellular elements of bone, cementum, dentin, and enamel decrease in significance in that sequence (FIGURES 1 to 9; TABLES 1 to 4).

(2) Histochemical observations suggest that *alkaline phosphatase* is conspicuously present in the osteogenic and odontogenic cells, and, to a lesser extent, in the calcifying matrix and the osteoclasts, suggesting that this enzyme may be involved in the whole life cycle of the mineralized tissues: formation, calcification, and resorption.The participation of other enzyme systems in one or more of these processes has not been extensively explored, but preliminary histochemical observations suggest that *oxidase* and possibly *dehydrogenase* enzyme systems may be involved. *Glycogen* has been demonstrated by the periodic acid-Schiff method in the osteogenic and odontogenic cells prior to calcification. Another Schiff positive component, attributed to a *carbohydrate-protein complex* has been demonstrated within the cytoplasm of active osteoblasts, cementoblasts, odontoblasts, and ameloblasts, as well as within the ground substance formed by these cells within the matrix of bone, cementum, dentin, and enamel. A metachromatic reaction accompanied by basophilia has been demonstrated (a) in the ground substance of the stellate reticulum of the enamel organ and in the interprismatic regions adjacent to the calcifying enamel prisms; (b) in the ground substance of the dental papilla and in the peripheral regions of the dentinal tubules, adjacent to the dentin matrix; and (c) in the ground substance of the dental sac and in the region surrounding the Sharpey's fibers of the cementum. These histochemical reactions suggest the presence of a *sulfated acid mucopolysaccharide* similar to that observed in cartilage, Wharton's jelly, nucleus pulposus, and cornea; structures which, like cementum, dentin, and enamel, are either avascular, acellular, or both. A positive histochemical reaction for *sulfhydryl groups* has been found in the odontogenic cells and pre-enamel matrix and, to a lesser degree, in the predentin. As dentin calcifies, the above reaction disappears, whereas the enamel reacts for *disulfide bonds*. A considerable portion of the organic constituents of all hard tissues consists of *citric acid*, and *peptide* material has been recovered even from the fully calcified, acellular enamel.

(3) In contrast to bone, with its microscopic landmarks of osteoblastic and osteoclastic remodelling, the cementum and dentin of functioning teeth are normally only subject to apposition in response to wear and tear—whereas the acellular and avascular enamel is completely protected from microscopi-

ally demonstrable remodelling. At the submicroscopic or chemical level of observation, the four mineralized tissues share a certain unity of organization nd, hence, of functional potentialities. It is suggested that *saliva and enamel* epresent a liquid-solid system similar in relationship to that existing between he extracellular connective tissue fluids and the mesenchymal hard tissues: one, cementum, and dentin. It is suggested, furthermore, that better understanding of these relationships may explain not only the protection which the lental hard tissues appear to enjoy, even in the presence of dynamic bone netabolism, but also certain pathological conditions to which the teeth are articularly prone, even in the absence of general bone pathology. Basic to hese problems is the nature of the linkage between the organic and inorganic onstituents of the four mineralized tissues, and the question as to what extent ne or more ingredients can be withdrawn or accrued selectively without imultaneous turnover of others (FIGURES 37 to 41, TABLES 5 to 7).

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Items of Discussion Related to this Paper

Doctor Leonard F. Bélanger (*Department of Histology, University of Ottawa, Ottawa, Ont., Canada*): With reference to the paper of Doctor Sognnaes entitled "Microstructure and Histochemical Characteristics of the Mineralized Tissues," Doctor Sognnaes has rightly stated that there is considerable disagreement among histologists as to localizations of metachromatic material. The problem lies mainly in the fact that there is no standard procedure for obtaining this reaction. While certain authors consider metachromatic only what they cannot remove by various usual treatments, I consider, with Pearse, that "in a true histochemical test no differentiation must be employed." When we have compared the metachromasia obtained by examination in distilled water or mineral oil with the autoradiographs of S^{35} introduced as SO_4 and retained in the tissues after the usual histological treatment, we have found a strict parallel between metachromatic areas and the autoradiographic record of what appears to be the sulfopolysaccharides (Anat. Record, in press).

Doctor Sognnaes: I should like to add a few comments regarding the evidence that sulfated acid mucopolysaccharides serve as the local factor which binds calcium phosphate to the organic matrix of the mineralized tissues. Both Doctor Sobel's and Doctor Leblond's presentations have made this concept an

attractive one, but there are a few histochemical and chemical observations which, if substantiated, might lead to a somewhat different interpretation.

The matrix *per se* of the mineralized tissues, while periodic acid-Schiff positive, does not appear to be sufficiently basophilic to suggest the presence of a sulfated acid mucopolysaccharide. The regions in which a combination of distinct metachromasia and basophilia have been demonstrated appear to be adjacent to, rather than within, the calcifying matrix (for example, certain regions within the dentinal tubules and between the calcifying enamel prisms). These are very narrow zones, which, even with the precise radioautographic technique developed and employed by Leblond, Greulich, and Bélanger, are practically impossible to distinguish from the matrix proper. To identify the radioisotope distribution within these minute regions, as contrasted with the matrix between, would require sections less than one micron thick. Even with such a technique, which we are in the process of applying to this problem (Bogoroch and Sognnaes, 1954), it is difficult to distinguish the small areas here involved, because of the relatively large size of the granules in presently available photographic emulsion.

In addition, it would seem that the radioautographic demonstration of internally administered radiosulfur within the mineralized tissues could be due to several sulfur combinations; namely the following: In the predentin region, our application of the new histochemical method of Barrnett and Seligman (1952) and Barrnett (1953) has revealed a reaction for SH groups, which, as dentin calcifies, disappears without being converted to SS bonds. This is in keeping with chemical evidence that there is no cystine in collagen (Bowes and Kenton, 1948). Within the walls of the dentinal tubules a strong metachromatic and basophilic reaction has been observed (Wislocki, Singer, and Waldo, 1948), suggesting the presence there of a sulfated acid mucopolysaccharide. Finally, there is in collagen the sulfur of methionine and, although this represents only 0.8 per cent of the collagen (Bowes and Kenton, 1948), it should be recalled that there is about 100 times as much fibrous protein in dentin as there is in enamel.

In the developing enamel, we have observed a distinct SH reaction, part of which appears to be transformed into SS bonds in the more mature enamel. The SH groups are attributed to cysteine, and the SS groups to cystine, of which there is less than 1 per cent in enamel keratin, as compared to over 10 per cent in hair and nails (Block, 1951). Furthermore, we have observed a metachromatic and basophilic reaction between the calcifying enamel prisms (Wislocki and Sognnaes, 1950). This reaction may be attributed to a sulfated mucopolysaccharide (SO_4), a portion of which may eventually become bound to the mineral component, although evidence is lacking. Finally, there is in enamel the sulfur of methionine (CH_3S) which, according to Block (1951), is present in similar amounts, namely, 1 per cent, in enamel, hair, and nail keratin alike.

In other words, there appear to be at least four different sources of organically bound sulfur in the enamel (SH, SS, CH_3S, SO_4), three in the dentin (SH, CH_3S, SO_4) and, presumably, the same in cementum and bone.

It is concluded from these observations: first, that the appearance of internally administered radiosulfur in bone, dentin, and enamel cannot be solely

attributed to the presence therein of a sulfated mucopolysaccharide; second, that this substance, even though present, is not necessarily located within the calcified matrix *per se*. On the other hand, the suggestion is offered that the sulfated acid mucopolysaccharide component, rather than being the local factor or key to calcification (*e.g.*, the linkage between the fibrous and crystalline elements), may serve to maintain certain regions in the uncalcified state, and provide metabolic pathways through these relatively avascular and acellular structures. These histochemical considerations are compatible with the chemical determinations made 20 years ago by Logan (1935), when he showed that the conversion of cartilage to bone matrix is characterized by a very marked loss of organic sulfate.

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THE ELECTRON MICROSCOPY OF ENAMEL AND DENTIN

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The principal findings during the first decade of electron microscopy of dental tissues have been summarized in a recent review publication.[1] In that paper, all of the available literature was cited, and emphasis was laid upon the structural variations which have been universally observed in normal, mature enamel and dentin. Some of these variations, like those which occur in the contours of enamel prisms when viewed in cross section, are not new to microscopists, for they have long been seen under the optical microscope. Other variations, such as those that exist in the organic matrix of the enamel, would not have been demonstrable except by use of the higher resolving power of the electron microscope. The impact of such observations with the electron microscope on existing histological concepts has been the realization that the structures of enamel and dentin are considerably more complex than was hitherto believed. Instead of leading directly to a clearer understanding of the basic structural units of enamel and dentin, these more detailed findings have increased the difficulty of interpretation.

In the present paper, some of the major areas in which information on the structure of enamel and dentin is still incomplete will be pointed out, and the contributions to present-day knowledge being made through investigations with the electron microscope will be discussed at some length.

The most fundamental problem in histological studies of enamel continues to be exact definition of the basic structural unit. There is little question today that mature human enamel is made up almost entirely of prismatic structures, extending individually from the dentino-enamel junction to the surface of the tooth and, for the most part, lying parallel to each other.[2] Investigations of developing enamel have indicated quite conclusively that each prism is laid down by a single ameloblast, which progresses outwardly in the course of enamel formation from a point of junction with the layer of odontoblasts to the contour line of the fully-formed crown of the tooth.[3] Taken together, such facts suggest that the prism is the structural unit of enamel. Even though the enamel prism has a diameter of *ca.* five microns, and is thus well within the limits of resolution of the optical microscope, it has not been possible to derive an accurate, all-encompassing description which can be applied to prisms in general. In the most widely accepted working concept of enamel structure, the prism is considered as a long filament, roughly hexagonal, round or scale-shaped (arcade-shaped) in cross section, surrounded by a thin organic sheath, and separated from neighboring prisms by varying amounts of cementing or interprismatic substance. The inadequacies of such a definition have long been realized, principally because of the marked differences in appearance between regions of hexagonal or round prisms and regions of scale-shaped prisms. In the former areas, the prisms appear well demarcated, entirely surrounded by sheaths and definitely separated from each other by interprismatic material. In the latter

areas, however, the prisms are not so well delineated. The prism sheaths appear to be incomplete, and interprismatic substance is not always visible as a separate entity. Various hypotheses have been offered for the arcade configuration. It has been proposed that the scalelike contour results from uneven compression during calcification, that the arcade-shaped prism is completely surrounded by a sheath, one portion of which is difficult to bring out under the optical microscope, and that, in regions of arcade-shaped prisms, there is no interprismatic substance.[2]

The principal factors which have limited progress in investigations of enamel structure with the optical microscope have been those inherent in the specimens prepared for examination. Most observations have been made on ground sections, which are too thick for optimal resolution when illuminated by transmitted light. More recently, studies of the surfaces of ground sections examined under reflected light have been encouraging, but as yet have not thoroughly elucidated the basic structure of enamel.[2] In the last few years, it has become evident that prism structure can be studied indirectly in sections of organic matrix which has been carefully recovered from artificially demineralized enamel.[4] The principal handicap in examinations of such material under the optical microscope is the close proximity of the sheaths of neighboring prisms, which are separated, at most, by about one micron of interprismatic substance. Adjacent sheaths are seldom resolved as individual entities.

The shortcomings of preparations used in optical microscopy, have been eliminated, to some extent, in those made by different methods for examination under the electron microscope.[1] Through the use of replicas of etched or unetched surfaces of ground sections, it has been possible to view enamel prisms in great detail in one plane. The fine structure of the organic matrix has become readily demonstrable in thin sections of artificially demineralized mature enamel and of either demineralized or undemineralized developing enamel.

The various contours of enamel prisms previously observed in cross sections under the optical microscope have also been noted under the electron microscope. Round, scalelike (arcade-shaped, three-sided), hexagonal, polygonal and very irregular outlines have been described.[1, 5, 16] Innumerable examples of configurations that must be explained before the basis structure of enamel can be understood are to be found among the micrographs of preparations made in this laboratory. A continuing search for prisms with uniform outlines and completely encircled by sheaths has met with surprisingly little success. As an example, among 2000 prisms shown in 500 micrographs made from replicas and thin sections of 185 specimens of enamel, only two per cent appeared hexagonal or round and showed complete sheaths. Of the remainder, all with incomplete prism sheaths, 57 per cent were arcade-shaped, 31 per cent polygonal or oval, and 10 per cent very irregular. It is becoming increasingly evident that the prism form most frequently encountered is arcadelike or scalelike. This configuration has been observed in every type of preparation that has been made. It has been noted in replicas of etched ground sections (FIGURE 1A), and in thin sections of demineralized mature enamel (FIGURE 1B), demineralized developing enamel (FIGURE 1C), and carious enamel not demineralized

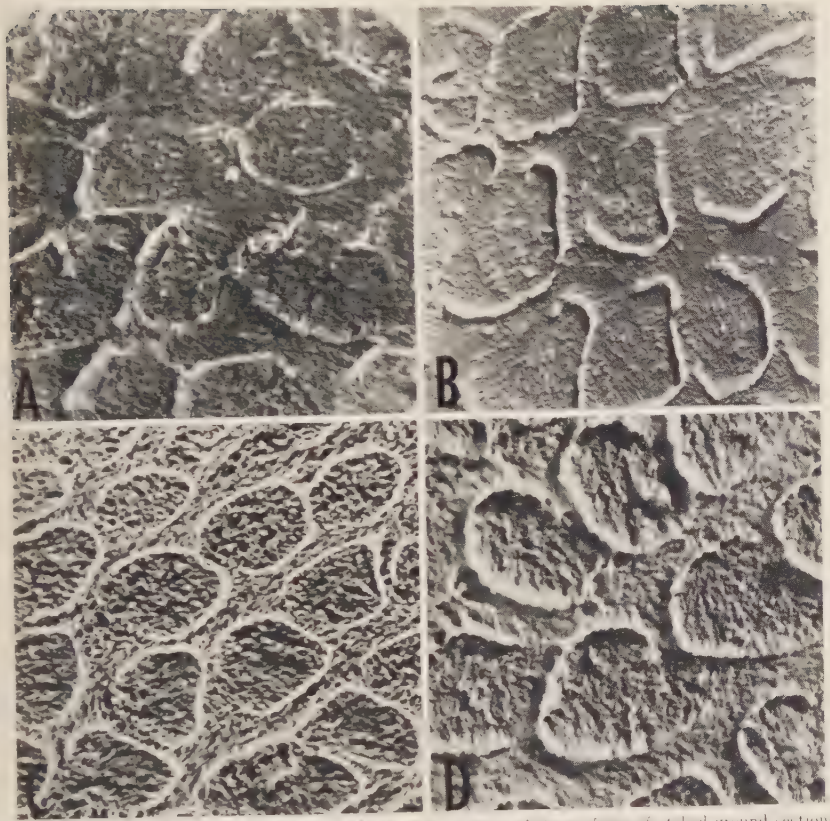

FIGURE 1 Typical arcade-shaped prisms observed in replicas of the surfaces of etched ground sections of normal mature enamel (A), and in sections of demineralized normal mature enamel (B), demineralized developing enamel (C), and decalcified enamel (D), fixed in the natural state without artificial demineralization. Magnifications: ×4000.

artificially (FIGURE 1D). In all instances, the prism sheath has appeared, in fact, to be incomplete, and it has been difficult to differentiate structurally between intraprismatic and interprismatic substance. It is of special interest that the arcade form has been observed in very immature enamel, which has undergone little or no calcification. This observation casts some doubt upon the explanation of the arcade shape on the basis of compression or uneven hardening during calcification.[3, 15]

A disturbing feature of enamel structure has been the apparent incompleteness of sheaths and, ofttimes, the lack of interprismatic substance in areas where the prisms are not arcade-shaped in cross section. Similar deficiencies have also been observed in sections cut obliquely or longitudinally through prisms. Close examination of the micrographs in FIGURE 2 reveals that, in some regions, a single band of sheath material seems to be shared by two adjacent prisms. There is also some evidence that, as previously suggested,[15] prism sheaths may be fused together and interprismatic substance may be absent (FIGURE 2D).

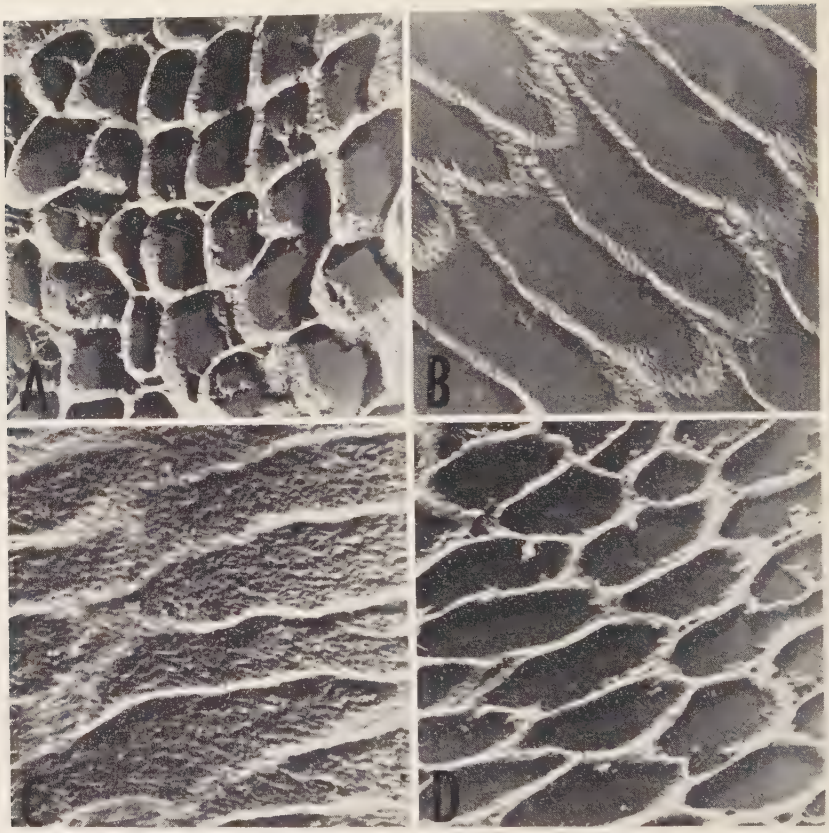

FIGURE 2. Deficiencies in prism sheaths observed in cross (A, D), oblique (B), and longitudinal (C) sections of demineralized mature enamel. Note close approximation of adjacent sheaths in (D). Magnifications: ×3600.

As indicated above, many of the prisms seen are very irregular in shape. The most common configuration, as yet inexplicable, has been one in which prisms appear to have projections extending between adjoining prisms (FIGURES 3A and 3B).[1, 15] The most bizarre configuration which has been seen often enough to warrant mention is one in which well-defined secondary structures seem to be present within the prisms (FIGURE 3C).

The extension in vision afforded by the electron microscope has offered the additional opportunity of discerning, for the first time, structures too small to be visible under the optical microscope. The most striking submicroscopic element thus far noted has been a fibrillar network which seems to constitute the basis of the organic matrix of the enamel (FIGURES 1A–D, 2A–D).[1, 7, 8, 11-15, 17-19] Although the fibrils can be found throughout the enamel (FIGURE 3D), future studies must be directed at determining their exact distribution, quantity, size, and orientation. A beginning has already been made in ascertaining the embryological origin of the fibrils. Investigations of enamel in various stages of development have indicated that the organic matrix is amor-

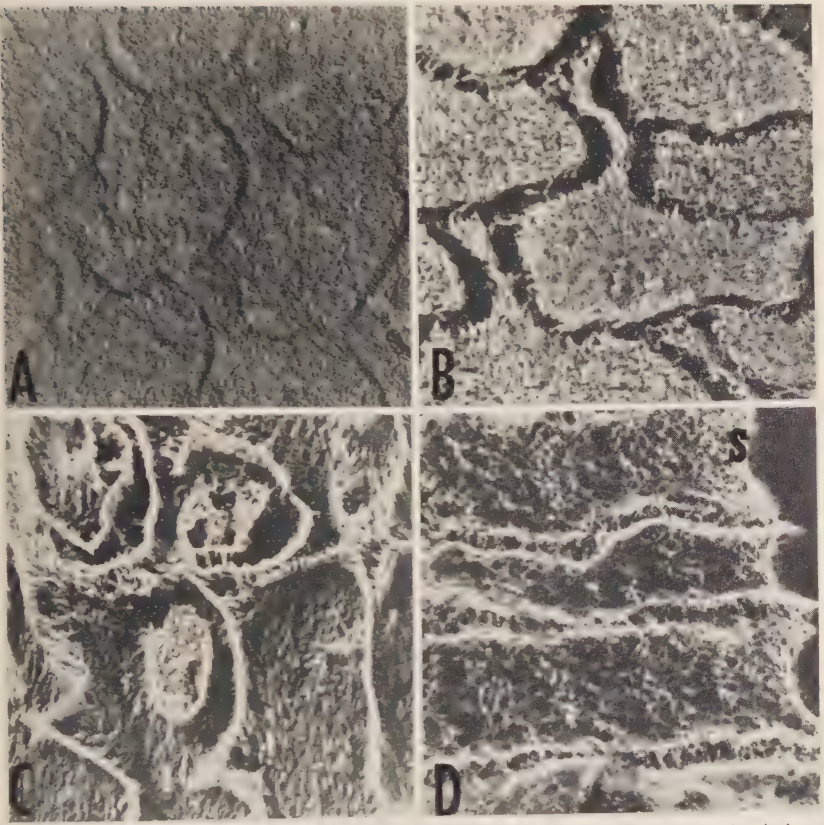

FIGURE 3 (A, B, and C). Irregular prism outlines in mature enamel observed in replicas of etched ground sections (A), and in thin sections of demineralized matrix (B and C). Note secondary structures apparently within prisms in (C).
(D) Typical fibrillar matrix in a section of demineralized subsurface enamel. Outer enamel surface is at S. Magnifications: ×6000

phous or homogeneous in character when first laid down by the ameloblasts, and gradually becomes fibrillar during calcification.[1, 20]

Although it has been possible to examine in great detail the organic matrix, a comparable degree of success has not been achieved in observing the submicroscopic crystals enmeshed in this matrix and constituting the main body of the enamel. Detailed information regarding the sizes, shapes, orientations, and quantitative distribution of the crystals must still be developed before the structure of enamel can be completely understood. Crystal-like objects have been observed in replicas of the surfaces of ground sections,[1, 7, 8, 10, 11, 12, 15, 21] but their individual outlines have not been distinct enough to permit more than generalized statements about their orientation. Within the prisms, the long axes of the "crystals" have appeared to be parallel to,[7, 8, 11] or at angles of 20 to 40 degrees to[21] the long axes of the prisms. In the interprismatic areas, the particles have been said to lie obliquely between prisms, often nearly at

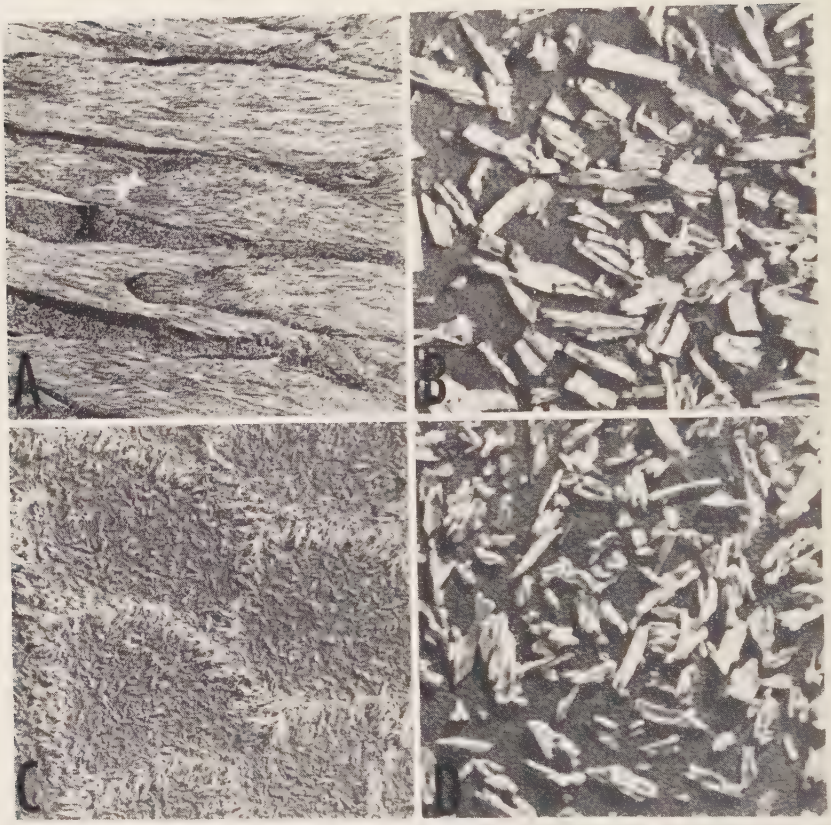

FIGURE 4. Crystal-like objects observed in sections of undemineralized developing enamel (A and B), partly demineralized normal mature enamel (C) and carious enamel sectioned in the natural state without artificial demineralization (D). Micrograph (B) represents a higher magnification of area X in micrograph (A). Magnifications: A, ×3000; C, ×6000; B and D, ×15,000.

right angles to them,[9-11] and to be more sparsely and unevenly distributed than within the prisms.[21]

Similar indications of crystal structure have been observed in replicas and in thin sections made in this laboratory. Some examples of crystal-like objects seen in such sectioned material are presented in FIGURE 4. It should be noted that, in longitudinal sections of developing enamel, there have been suggestions that the long axes of the crystals may be roughly parallel to those of the prisms (FIGURE 4A). In cross sections of partly demineralized mature enamel, the crystals at the peripheries of the prisms have appeared to be at right or oblique angles to the long axes of the prisms (FIGURE 4C). It has thus far been difficult to determine the relationship between the crystal-like structures in these latter areas and the prism sheaths and interprismatic substance seen in sections of completely demineralized enamel.

A great deal of variation has been observed in the size of the crystal-like objects noted in sectioned enamel. Since none of the sections were of fully mature

enamel, and because there is a possibility of fragmentation during sectioning, it would seem unwarranted, at this time, to present estimations of the dimensions of the crystals. Similar variations in the size of these particles have been found, however, in replicas and comminuted samples of mature enamel. Reported lengths have ranged from 600 Å. to 10,000 Å.[1, 7, 10, 18, 22]

The foregoing descriptions of the results of studies of enamel with the electron microscope demonstrate the great potentiality of this histological tool. It is clear that a substantial beginning has been made toward the development of more precise information on the structure of enamel. In the following part of the discussion, which deals with investigations of dentin, it will be noted that somewhat less progress has been made. This fact has been especially true with regard to the structure of the organic matrix, which has not been easy to observe in fine detail, and with respect to the inorganic, crystalline component, which has remained virtually unseen. A noteworthy start toward a better understanding of dentin structure has been made, but much work remains to be done.

It is difficult to consider dentin in terms of basic structural units. In contrast to enamel, which is in essence a mass of closely packed prismatic units, dentin consists essentially of a calcified fibrillar matrix, permeated by tubular channels in which are found long processes emanating from cells not present within the matrix itself. The tubules and their contents, however, are repetitive throughout the tissue and, in that sense, they may be regarded as homologous units of structure, the exact definition of which is essential to an understanding of the morphological characteristics of dentin.

As a result of earlier optical microscopy, three major questions about the dentinal tubules remain unresolved.[3, 25, 24] Two of these questions concern the nature of the odontoblastic process (Tomes' fiber, dentinal fiber) which is located within the confines of the tubule. There has been considerable controversy as to whether the process is solid or tubelike, and whether or not it fills the entire lumen of the tubule. The third question concerns the existence of a special lining of the tubule (Neumann's sheath).

Under the electron microscope, odontoblastic processes have shown several different appearances. They have most often looked thin-walled and tubelike (FIGURE 5A).[1, 5, 7, 11, 21, 24, 27] Recent observations in this laboratory have indicated, however, that on occasion the processes may appear solid (FIGURE 5B). Several workers have noted what seem to be additional fibers enclosed within the membranelike outer wall of the process (FIGURE 5C).[1, 12, 28, 29] It has been reported that the processes are bundles of collagen fibrils showing typical cross striations,[10, 30] but the accuracy of this finding is open to considerable doubt. Bizarre configurations, such as one in which there appear to be several odontoblastic processes in one tubule, have also been observed (FIGURE 5D).

Little evidence has been accumulated thus far regarding the amount of space the odontoblastic process occupies within the tubule. There have been a few indications that some shrinkage of the process may occur during specimen preparation.[1, 15, 24, 31]

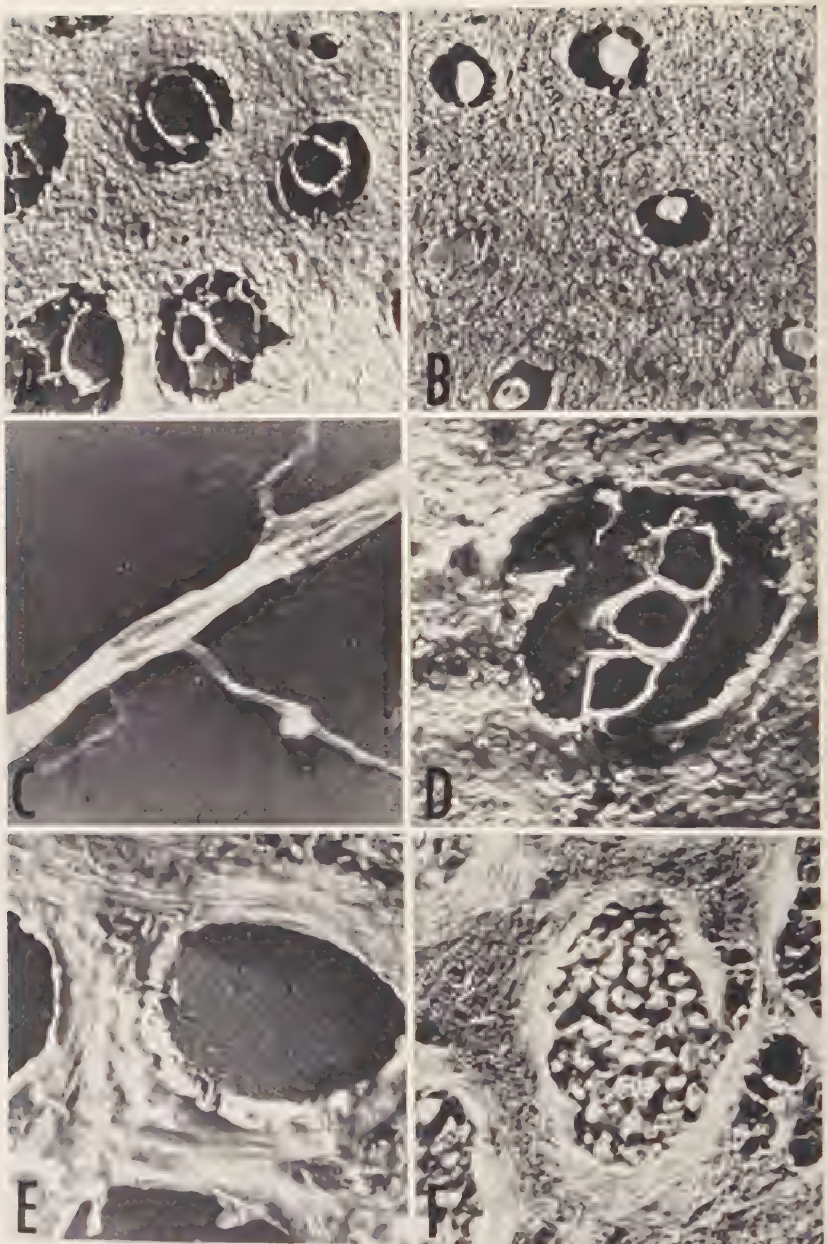

FIGURE 5 (A and B) Thin-walled (A) and solid (B) odontoblastic processes observed in sections of demineralized dentin.

(C) Apparent presence of additional fibers within an odontoblastic [illegible]. [illegible]

(D) [illegible] dentin.

(E) Collagen fibrils observed in a section of demineralized dentinal matrix.

(F) [illegible] at the periphery of a tubule, observed in a section of [illegible] within tubule.

Magnifications: A and B, ×3000; C and F, ×6000; D, ×10,000; E, ×13,000.

It is the consensus of workers using the electron microscope that a definite membranelike lining of the wall of the dentinal tubule does not exist. Differences in the nature of the matrix at the border of the tubule have been observed, which may account for the semblance of a "sheath" in sections examined under the optical microscope.[1, 15, 18, 21, 26, 32] Convincing evidence has been presented that the fibrils of the matrix may have a circumferential orientation in this region, in contrast to the trellislike arrangement typical of intertubular areas.[1, 18, 26, 32] Increased density of organic material, a higher degree of mineralization, and differences in chemical composition have also been suggested as characteristics of the matrix immediately surrounding the tubule.[10, 18, 21] Recent studies of sectioned carious dentin in this laboratory have provided additional support for the ideas that the organic matrix may be more dense, and the fibrils oriented circumferentially at the periphery of the tubule (FIGURE 5F).

In connection with the features of the dentinal matrix mentioned above, it should be noted that, thus far, studies of the fibrillar component have not been particularly rewarding. The fibrils, which are submicroscopic in dimension, have been objects of special interest in electron microscopy, but it has been possible, only occasionally, to reveal them clearly and in large numbers. The difficulty has seemed to be due to the persistent presence of large quantities of an amorphous organic material in which the fibrils appear to be imbedded.[1, 15, 18, 32] This amorphous organic material may be the ground substance described earlier by optical microscopists. The fibrils which have been seen distinctly have been characterized by the cross striations at 640 Å. intervals which are typical of collagen (FIGURE 5E).[1, 15, 31, 32]

The problems in interpretation of the varied findings on the structure of enamel and dentin are readily recognizable. It is tempting to attribute the different appearances shown by a single element, such as the odontoblastic process or the prism sheath, to post-mortem changes or damage during specimen preparation, but, at present, there is little or no evidence available which might lend strong support to such explanations. On the other hand, it may be that the so-called basic structural units of enamel and dentin are actually multiform. The most challenging implications of such a possibility are the preclusion of simplified or stereotyped definitions of morphological characteristics, and the enormous task that will be involved in relating the structure of the parts to that of the whole.

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THE CHEMICAL NATURE OF THE ORGANIC MATRIX OF BONE, DENTIN, AND ENAMEL*

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The purpose of this review is to bring together results of recent biochemical analyses of normal human bone, dentin, and enamel. Studies on the recognizable structural elements by electron microscopy and histology invite the analyst to try to achieve a separation of these tissues before determining their chemical properties. Separation would be of special importance in the case of enamel, but the technical difficulties involved are considerable. These difficulties are due to a number of factors, such as the properties of the three types of tissue in regard to the cellular elements and to remodelling.

Other factors also require consideration when studying the amount of the organic matrix present in each tissue. Considerable variation in the degree of calcification is observed along bone and dentin, longitudinally, and bone Ca N ratios increase markedly until adult life. Crown dentin samples from deciduous and permanent teeth show the same degree of calcification. Such crowns differ, however, in enamel organic content, but no correlation with type of tooth or with age was observed for dentin or enamel analyses.

Summarizing the ranges of values, it is apparent that the organic content of bone normally ranges from 24 to 26 per cent, the values being 19 to 21 per cent in the case of dentin. With an organic content of 0.5 to 0.9 per cent, deciduous enamel differs significantly from permanent enamel, which has 0.4 to 0.8 per cent. The corresponding coefficients of variation are found to be about 4 per cent for bone and dentin, and 12 per cent for both types of enamel. TABLE 1 shows values calculated lately from the groups of samples analyzed by Hodge and his co-workers,[1] Dragiff and Karshan,[2] Cruickshank,[3] Le Fevre and Manly,[4] Armstrong and Brekhus.[6] The fifth and seventh entries[6, 7] relate to the present studies; the difference between these organic content values was shown to be due only to the higher calcification of the crowns.

The values for bone have been calculated from the nitrogen determinations described by Vogt and Tönsager,[5] and by Baker, Butterworth, and Langley,[9] who agreed on the nitrogen content of 4.7 per cent. Both sets, each of about 30 samples, show a distribution which does not appear to be quite normal, probably because of the continued calcification with age. Those for dentin and enamel, comprising four groups of 50 samples, appeared normally distributed with no age correlation.

Bone

Referring specifically to bone, it is apparent from the work of Baker and his colleagues that, if cancellous bone can be freed from intratrabecular material, its organic content does not differ from that of cortical bone. There are, how-

* The author's studies incorporated in this review were completed during the tenure of a Dental Research Fellowship in the Department of Dental Medicine, Guy's Hospital, London, England.
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TABLE 1

MEAN VALUES AND COEFFICIENTS OF VARIATION FOR THE TOTAL ORGANIC OR PROTEIN CONTENT OF DENTIN

Sample type	Analysis	Number of samples	Organic content	Coefficient of variation
(1) Total permanent	Incineration	48	28	4
(2) Root permanent	Incineration	37	26.7	4.1
(3) Root permanent	Incineration	41	26.2	5.4
(4) Total permanent	Extraction	21	18.6	4.5
(5) Crown deciduous	Wet combustion	50	16.5	4.1
(6) Total permanent	Nitrogen	20	19*	4.2
(7) Total permanent	Nitrogen	50	19.5*	4.4

* Protein content calculated from nitrogen values (factor 5.4). These two values and the value for mean organic content of deciduous dentin do not include combined water.

ever, differences between various types of bone in this respect, as found also by Strobino and Farr,[10] who suggested that bones should be sampled at points where nitrogen content was minimal. These points occurred in the epiphyseal regions of the shafts of the young cattle bones studied. Cross sections of bone at such points are probably the most suitable samples (FIGURE 1).

This is evident from a recent paper by Rogers, Weidmann, and Parkinson.[11] They showed that calcification increased rapidly up to the age of 25 and more slowly thereafter. These authors stressed the direct relation between nitrogen content and the degree of modification of the basic bone matrix by penetrating blood vessels. These noncollagenous elements contribute material richer in phenolic substance and material not rendered soluble by autoclaving. The quantity of these elements is so low that it is possible to state definitely that variation in the Ca N ratio corresponds to true differences in calcification. In fact, Rogers and his co-workers found that 90 to 96 per cent of the nitrogen in human, rabbit, and ox bone samples was collagenous (TABLE 2). From this proportion and from the total nitrogen value of 4.7 per cent, it is apparent that the collagen content of bone is about 23 per cent. Rogers found that 2 per cent of the nitrogen was in soluble form.

Recent work by Hamilton[12] indicates that a considerable proportion of the protein of finely ground bone is readily soluble in water. More could be extracted by buffer, this fraction then being precipitated by dialysis. The other protein of bone is the "osseoalbumoid" described 50 years ago by Gies and his co-workers. This protein was thought to be elastin by them and by Rogers, who considered that it accounted for 2 to 8 per cent of the nitrogen of shaft bone but for only 1 per cent of cancellous.

Rogers[13] digested the protein of demineralized bone with trypsin and purified the resultant mucopolysaccharide fraction. There appears to be about 0.4 per cent of this type of compound in bone, but chloride-carbonate extraction might yield more than this. According to Weidmann,[14] sphingomyelin, which is thought to promote calcification *in vivo*, is the chief substance present in fractions prepared by extraction with lipid solvents, the total accounting for 0.1 per cent of large ox bone samples. Lime extractions of ox femora powder yielded 0.24 per cent of a complex containing 70 per cent protein; amino sugars

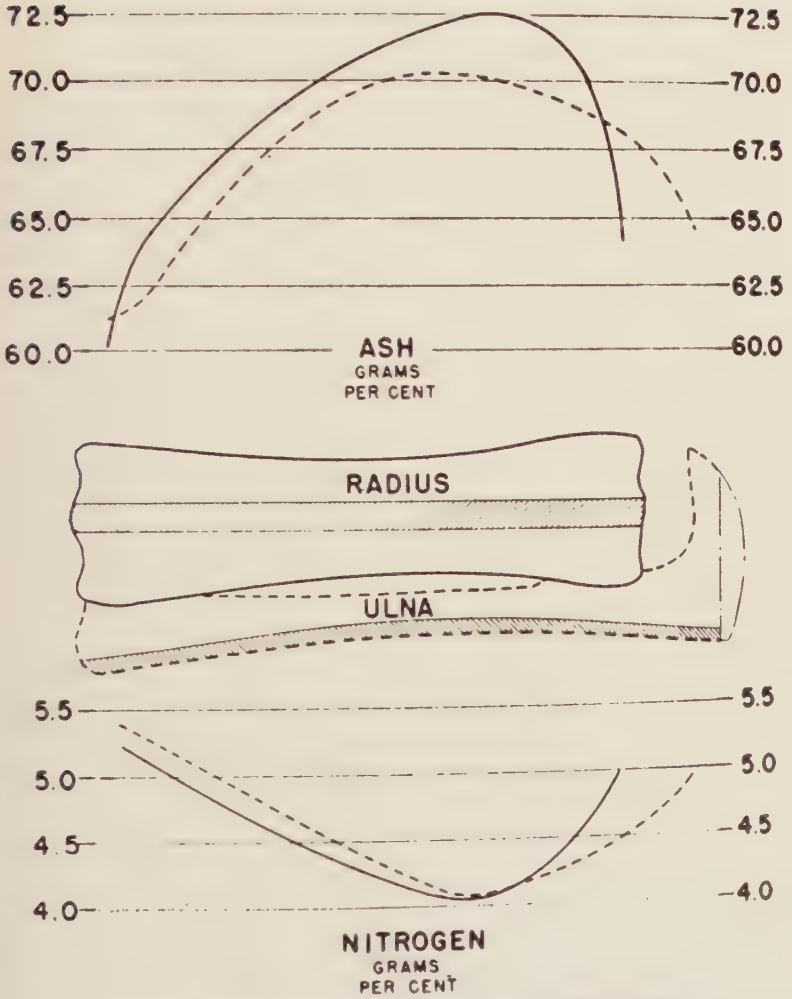

FIGURE 1. Nitrogen ash content along bovine bones (Strobino and Farr[10])

accounting for two fifths of the remainder, the other three fifths being galactose, mannose, and glucuronic acid (Eastoe and Eastoe, 1954).

Dentin

Studies on the organic constituents of the dental tissues have recently increased in number, as is apparent in the latest review on the biochemistry of the teeth.[16] Dentin is recognized as a fibrillar calcified collagenous matrix traversed by odontoblastic processes composed of a proteinlike elastin. Quantitative determination of the main protein is most satisfactory by an isolation procedure, although the proportion is so high that correct values can be deduced from the nitrogen content which is between 3.4 and 3.5 per cent. When

TABLE 2
COLLAGEN CONTENT OF HUMAN FEMURS
(Rogers, Weidmann, & Parkinson, 1952)

Age of subject	Total nitrogen %	Nitrogen (% of total nitrogen)		
		Soluble (A)	Insoluble (B)	Collagen (C)
2.5	4.83	2.1	4.2	96
7	4.97	1.9	0.8	96
10	4.93	2.1	4.0	90
15	4.78	1.4	2.9	92
27	4.73	1.8	1.4	95
41	4.65	2.0	3.2	94
65	4.67	2.2	6.7	88

A. Found in decalcifying fluid.
B. Not rendered soluble by autoclaving.
C. Note inverse relationship to column B.
A + B + C account for 96–102% of total nitrogen.

pulverized pooled samples of dentin are demineralized with 10 parts of normal hydrochloric acid in the cold, the yield of collagen varies from 17 to 18 per cent.

Under optimum temperature conditions, similar yields of collagen can also be obtained by demineralizing with neutralized ethylenediamine tetracetic acid (pH 7.5), or with phosphate-citrate buffer at pH 3. As in the case of preparations with mineral acid, 3 to 5 per cent of the total protein appears in soluble form. About one tenth of this fraction appeared to be noncollagenous, according to tyrosine and hydroxyproline analyses and about one third was diffusible. It was not possible to separate the two types of protein by electrophoresis on filter paper, and immunological tests did not disclose serum protein.

Lower yields of collagen were reported by Hess and his colleagues,[17] who demineralized ground dentin at room temperature. They recovered 98 to 99 per cent of the nitrogenous components, 75 to 77 per cent as insoluble protein, 14 to 15 per cent as soluble protein, and 5 per cent as diffusible components. Hydroxyproline was determined in bones and teeth by Neuman and Logan[19] who suggested that the collagen content of the mineralized tissues could be calculated from such values. Other amino acids in dentin have been investigated qualitatively by Atkinson and Matthews[20] and by Block, Horwitt, and Bolling.[21] Hess and his colleagues[18] determined the amino acids of dentin protein quantitatively by microbiological assay, confirming its collagenous nature.

However, one fortieth of the protein of dentin is resistant to autoclaving. Analyses show 12 per cent nitrogen, 5 per cent tyrosine, and 0.3 to 0.6 per cent hydroxyproline. Semiquantitative paper chromatography indicated the close resemblance between this protein and the similar residues from skin and bone collagens. However, elastin was clearly differentiated from these compounds by this method. Differences between skin and dentin collagens (TABLE 3) have not been noted in analyzing for nitrogen, hydroxyproline, carbohydrate, and hexosamine; the acid titration curves also followed the same course. The citric acid content observed by Zipkin and Piez[22] was confirmed, and the variability

TABLE 3

COMPARATIVE ANALYSES OF SKIN AND DENTIN COLLAGENS AND OF "DENTIN COLLAGEN RESIDUE"

Analysis values per cent	Purified skin collagen	'100/200-Mesh' dentin collagen	Dentin collagen residue
Nitrogen	18.4	18.4	11.8 to 12.0
Hydroxyproline	14.0	14.0	0.3 to 0.6
'Tyrosine'	1.4	1.4	4.8 to 5.2
'Lactose'	1.0	1.0	—
Hexosamine	0.28	0.25	<0.1

was seen to be similar to that of the total organic content, allowing for analytical variability.

It was then possible to find whether or not all of the significant organic constituents of lipid-free dentin had been recognized. A wet-combustion technique was used for this purpose. The amounts of the dichromate-sulfuric acid reagent required to oxidize the gelatin and its breakdown products, also the citric acid and the mucopolysaccharide, in the acid-soluble fraction were found to equal that actually needed to oxidize the total organic matter in the fraction. The organic constituents of normal pooled dentin were thus found to be: collagen, 18 per cent; insoluble protein residue, 0.2 per cent; mucopolysaccharide, 0.2 per cent; lipid, 0.2 per cent; citric acid, 0.9 per cent.

Hess and his colleagues have also studied the mucopolysaccharide[23] and lipid[24] components of dentin. Their application of a chloride-carbonate extraction process to dentin resulted in a 0.64 per cent yield of chondroitin sulfate. Cholesterol and its esters were prepared in a yield of 0.4 per cent by extraction. Soyenkoff, Friedman, and Newton[25] extracted 300 mgm. of lipid, mostly aminophospholipid, from 300 g. mixed dental tissues.

Enamel

Recent studies have drawn attention to the likelihood that the organic matrix of enamel is involved at an early stage in caries. The most important organic constituent is thought to be a keratin, as suggested by Hoppe a century ago. However, the amount of this insoluble protein in a full human dentition appears to be no greater than the keratin represented by a full-grown human hair. Histological evidence on structural composition is invaluable in deciding what comprises representative enamel.

Losee and Hess[27] give a very satisfactory account of the distinctions which should be recognized in preparing enamel for analysis of the organic matrix. These authors considered that "enamel proper" was bounded by lesser thicknesses of "inner enamel" and "outer enamel." Cuticle, pits, and grooves must be considered in connection with "outer enamel." Losee and Hess prepared a number of enamel samples of the various types ranging from "enamel proper with pits and grooves ground out" to "stained enamel with deep pits and grooves." The organic content of their samples ranged correspondingly from

TABLE 4
YIELDS OF PROTEIN FROM SEVERAL TYPES OF ENAMEL
(Losee & Hess, 1949)

Material analyzed	Yield %
1. Total enamel, stained, pits and grooves deep	0.49
2. Total enamel, unstained, pits and grooves deep	0.39
3. Total enamel, unstained, pits and grooves minimal	0.30
4a. Enamel proper, pits and grooves minimal	0.22
4b. Enamel proper, pits and grooves removed	0.21

0.2 to 0.5 per cent (TABLE 4). Pincus[26] reported analyses of the organic frameworks prepared from Nasmyth's membrane and the grooves of molars.

As dentin contains 40 times more organic matter than enamel, it is necessary to begin biochemical study of the latter tissue after purification until the dentin content has been reduced to about 0.2 per cent. It is possible to prepare samples of this standard by grinding the dentin away so as to leave only a few tenths per cent dentin, but the method is very laborious when large samples are required. Losee and Hess used this technique with success, as did Anderson.[29]

A more convenient method is that suggested by Manly and Hodge, in which pulverized tooth substance is stirred with bromoform-acetone mixtures of specific gravity such that the dentin floats from the heavier enamel. Repetition of the process yields 99.8 per cent pure enamel.

Many authors have attempted to measure the organic content of mature enamel by weighing before and after incineration or alkaline-glycol extraction. Nitrogen values given by Karlström[28] indicate that only one twentieth of the weight lost during incineration could be ascribed to the 0.3 per cent protein he believed to be present. Hodge and colleagues used both extraction and incineration procedures in parallel, and their values showed that the mean organic content plus water amounted to 4 per cent of the weight of the enamel sample. As the nitrogen content of the protein or proteins of enamel is indefinite, calculation of the protein content of enamel from total nitrogen determinations is unsatisfactory. However, there is a good agreement between the recent values for nitrogen content given by Anderson[29] as 0.077 per cent and by Losee and Hess as 0.073 per cent from an enamel sample with few pits and grooves. A protein content below 0.5 per cent was indicated from these values and from the pooled enamel sample of known purity and minimum nitrogen content examined by Deakins and Volker.[30]

The basic amino acids and cystine contents of the insoluble protein of enamel have been determined by Block, Horwitt, and Bolling,[21] also by Losee and Hess.[27] Both confirmed that the cystine content was much lower than that of other keratins. The first group of authors examined the fraction resisting pepsin and chymotrypsin treatment. They also determined the other amino acids qualitatively by paper chromatography. Another recent study by Hess and his colleagues revealed the presence of 5 per cent hydroxyproline in freshly-isolated insoluble enamel protein. Amino acids not previously determined were assayed microbiologically. Nitrogen represented by the values obtained

for 20 amino acids accounted for 98 per cent of that present in the protein analyzed. Similar values have recently been obtained[55] with freshly-isolated protein prepared from bromoform-separated enamel, but after removal of collagenous material, rather different analytical values were obtained. However, 4 per cent hydroxyproline was still present, as determined in the first fraction, on elution chromatography from a sulfonated polystyrene resin.

No soluble protein was revealed during these studies. However, Anderson noted that when enamel was demineralized with various acids, only about half the total nitrogen was present in the filtrate. From the histological field, it may be observed that Frisbie, Nuckolls, and Saunders[32] have indicated that the endpoint of demineralization needs to be controlled carefully in order to avoid some of the protein going into solution.

Soluble protein, however, was prepared readily from large batches of bromoform-separated enamel. This enamel was prepared in 97 per cent yield by bromoform flotations, four being required in most cases. It was shown that the organic content of enamel did not depend on the age or type of teeth, and that sound enamel from carious teeth had the same organic content as that from intact teeth (TABLE 5). The 20 batches, each of 10 grams, were all prepared by the bromoform flotation method. Dentin content was checked by microscopic counts which were correlated with analyses by wet combustion. Such batches of enamel, 99.8 per cent pure, were packed in cellophane bags and dialyzed against 0.5 per cent phosphoric acid. Yields of soluble and insoluble proteins are shown in TABLE 6. These proteins were isolated, at first, without further purification in order to determine how much dichromate-sulfuric acid reagent was required to oxidize them. The relationship was such that no numerical conversion factor was necessary in expressing the chemical microequivalents on a weight percentage basis. Later, it became possible to examine a diffusible fraction, at the expense of a reduction by one tenth in the yield of insoluble protein, demineralizing enamel with 25 per cent phosphoric acid (FIGURE 2). The three fractions, insoluble and soluble protein, together with the diffusible

TABLE 5

ORGANIC CONTENTS OF ENAMEL BATCHES FROM SOUND AND CARIOUS TEETH

Enamel from sound teeth		Enamel from carious teeth	
Organic %	Dentin %	Organic %	Dentin %
0.56	0.10	0.52	0.08
0.61	0.16	0.66	0.14
0.62	0.10	0.56	0.08
0.60	0.14	0.68	0.04
0.59	0.20	0.62	0.05
0.59	0.12	0.52	0.03
0.56	0.25	0.68	0.10
0.60	0.19	0.66	0.06
0.65	0.16	0.58	0.08
0.63	0.08	0.55	0.16
Means.....0.60	0.14	0.60.	0.08

TABLE 6
YIELDS OF PROTEINS FROM ENAMEL

Soluble protein yield %	Insoluble protein	
	Yield %	Nitrogen %
0.17	0.20	13.3
0.17	0.16	13.5
0.19	0.17	13.2
0.18	0.16	13.2
0.17	0.22	13.4

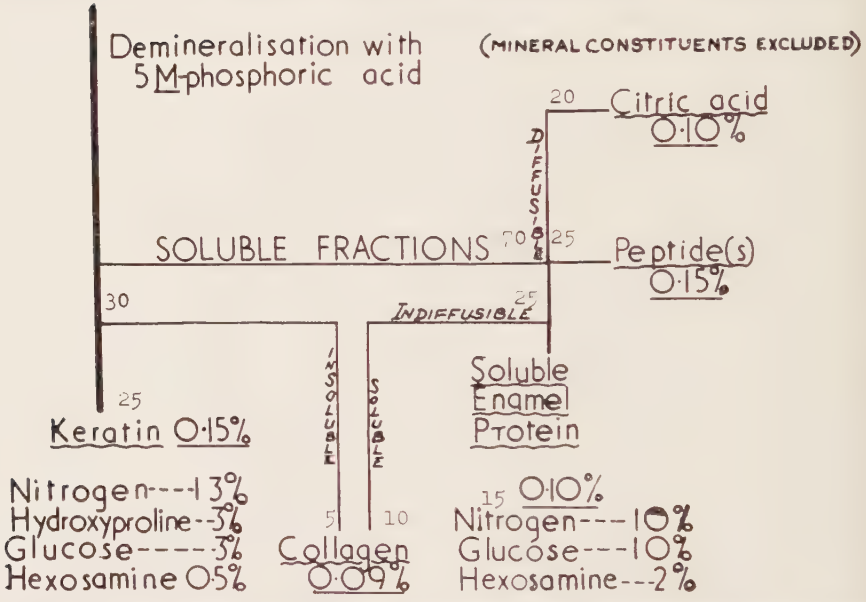

FIGURE 2. Fractionation and analysis of enamel constituents (Stack). The smaller figures are percentages of the total organic content appearing in the fractions.

fraction, were analyzed with the dichromate reagent. Including the 0.10 per cent citric acid content, the sum was found to agree with the value obtained by the wet-combustion of purified enamel before demineralization. The diffusible fraction was recognized as peptide in nature by extraction into butanol and analysis with a ninhydrin reagent, also by eluting similar material from columns of pulverized enamel. Such a reagent had been used by Hutton and Nuckolls[35] in locating peptides and amino acids near the enamel surface of unerupted molars. It was noted that the peptide content was correlated with the organic content of the enamel in which it was determined (TABLE 7). The carbohydrate and mucopolysaccharide contents of the protein fractions were determined also. The methods involved digestion with enzymes or boiling with water, in the case of the insoluble protein; and by electrophoresis on filter paper, in the case of the soluble protein. The minimum hydroxyproline content of

Table 7
Peptide Content of Enamel

Organic %	Peptide %	Organic %	Peptide %
0.54	0.12	0.60	0.17
0.53	0.13	0.66	0.19
0.57	0.13	0.69	0.19
0.65	0.15	0.70	0.19

purified insoluble protein was about 2 per cent, a value 10 to 15 times more than is apparent in the proteins of fingernail and hair respectively. These three proteins were also compared by semiquantitative paper chromatography. Similar hydroxyproline content was noted for the noncollagenous fraction separated from the soluble protein concentrate by electrophoresis. The purified fraction contained 10 per cent nitrogen and 2 per cent hexosamine, and 10 per cent hexose. This compound was apparently glucose, but Egyedi[34] has indicated the presence of glycogen. The protein thus ranks as glycoprotein. The hexosamine and glucose contents of the insoluble protein were 0.5 per cent and 3 per cent after purification. At this stage, further study of the soluble protein and peptide fractions is necessary. It is tempting to suggest that it is not the keratin, but these constituents, which are of importance in the proteolytic processes involved in dental caries.

Summary

Direct determinations indicate that "average" bone contains 24 to 26 per cent organic matter. Corresponding values for dentin from deciduous and permanent teeth are both 19 to 21 per cent, but there is a significant difference between the values for the enamels (0.5 to 0.9, and 0.4 to 0.8 per cent). The coefficients of variation are 4 per cent for bone and dentin, 12 per cent for enamel.

Bone calcification increases with age throughout life, and the degree of calcification appears related to the proportion of collagen (90 to 96 per cent) in the total matrix. Apart from collagen, and also noncollagenous protein (averaging 5 per cent in shaft bone), the only other organic constituents of importance in lipid-free cleaned bone are mucopolysaccharide and citric acid.

Dentin calcification varies longitudinally over a considerable range, but does not depend on age or type of tooth, as found also for citrate content (0.9 per cent). Only 3 to 5 per cent of the nitrogenous constituents are water-soluble, and 2 to 3 per cent of the remaining collagenous matrix resists autoclaving, but can be differentiated from elastin. Mucopolysaccharide can be extracted by buffers, the yield of 0.6 per cent being greater than that suggested by analysis.

Enamel contains 0.2 per cent of a eukeratin for which the amino acid composition has been evaluated before and after purification. Hydroxyproline is present after removal of the associated collagenous material. Soluble glycoprotein has been prepared in similar yield and a somewhat smaller peptide fraction studied. Including citric acid (0.1 per cent), these constituents account for the total organic content of enamel.

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CRYSTAL-COLLAGEN RELATIONSHIPS IN BONE AS OBSERVED IN THE ELECTRON MICROSCOPE. III. CRYSTAL AND COLLAGEN MORPHOLOGY AS A FUNCTION OF AGE*

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Previous electron microscopic observations of bone have been reported. The size and shape of bone crystals and collagen-crystal relationships in fragmented bone were demonstrated by Wolpers in 1943 and by Robinson in 1952 (FIGURE 1). Electron micrographs of sections of undecalcified and partly decalcified bone demonstrated the crystal-collagen relationships, and electron diffraction patterns of these same sections showed parallel alignment of the c-axis of the unit cells of the bone crystals to the long axis of the collagen fibers. Sections of decalcified bone demonstrated the relative space occupied by cells, canaliculi, and collagen fibers, as well as some detail of the collagen fibers (Robinson and Watson, 1952; FIGURE 17).

Synthetic basic calcium phosphate crystals were studied, at various stages during their development, by electron microscopy and electron diffraction (Watson and Robinson, 1953). It was shown that these crystals have the c-axis of the unit cell in the long crystal axis, and that the crystals are tabular in habit, so that the c-axis parallels the broad crystal surfaces.

Analyses of bone usually divide it into three major components: organic, inorganic, and water. The inorganic and water components have shown most variability during the process of bone maturation. For instance, Vogt (1949) and Vogt and Toensager (1949) investigated the marrow-free cancellous bone of the human ilium. They found that, on the basis of 4 samples each from 16 cadavers in different age groups, the ratio (dry substance fresh nondehydrated bone) increased with age from "slightly under 70 per cent at birth to about 80 per cent in old age." In other words, the average water component decreased from 30 per cent to 20 per cent by weight as the human ilium aged.

Hammet (1925) after analyzing rat bone stated, "The percentage of water of both bones (humerus and femur) of both sexes decreases with age. This is largely due to ash deposition. The increase in percentage of organic matter is a minor factor."

Burns and Henderson (1936a and 1936b) noted a decrease in water content with increasing age of puppy bone.

In a related field, Deakins (1942) studying developing enamel measured the organic, inorganic, and water content of this hard tissue and found that *per unit volume*, the water content decreased while the inorganic component markedly increased. The organic component decreased during the *very* earliest stages of development and then remained constant. He interpreted his data

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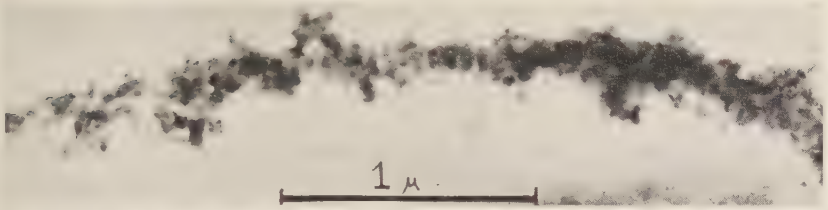


FIGURE 1. Collagen fiber encrusted with crystals from the cortex of the human rib. Following agitation of shavings of fresh bone in a Waring Blendor, the aqueous mixture was held at pH 8 by a phosphate buffer. Streptococcal hyaluronidase was added and the mixture was incubated for three hours. The bone was agitated a second time in the blendor and a drop of the cloudy mixture placed on an electron microscopic specimen screen. Periodicity is clearly seen along part of the object. The periodically recurring bands are interpreted as aggregates of inorganic bone crystals attached at regular intervals along the underlying collagen fiber. Magnification ×33,000. Courtesy of "Journal of Bone and Joint Surgery." (Robinson, 1952).

as showing that the volume first occupied by water was almost exactly replaced by inorganic crystals as the enamel matured. Unpublished data of a similar type being obtained at present by Robinson (1953) suggest that the organic as well as the inorganic volume increases somewhat with age at the expense of the water volume in human bone.

In general, then, newly formed bone is more hydrated than older bone. Unless disease alters the relative rates of bone resorption and deposition throughout the skeleton (Amprino, 1953b), there is a larger amount of newly formed bone in newborn animals and a larger amount of old or well-established (less hydrated and more completely calcified) bone in older animals. This accounts for Hülsen's (1898) finding that the specific gravity of fresh human bone varied between an average of 1.67 in the newborn to 1.86 in the adult.

The relative age of any portion of a bone is apparently associated with its physiology. This association has been emphasized by studies of exchange of calcium and phosphorus isotopes and uranium with bone. Autoradiographic and historadiographic techniques have demonstrated less calcific density in newly formed than in established bone (Amprino and Bairati, 1936, Amprino, 1952, Amprino and Engström, 1952); differences in the X-ray density due to varying proportions of cement substance and collagen fibers in adjacent bone areas (Amprino and Engström, 1952); and, finally, greater uptake of radioactive calcium and phosphorus by newly formed, as contrasted with longer established, bone areas (Amprino, 1952a, b, c).

Uranium is taken up in greater proportion by new than by old bone, although "the differential uptake in recent as compared to old bone tissue is much greater for radioactive calcium than for uranium" (Amprino, 1953a). The skeletal localization of radium suggests that it, too, is concentrated almost entirely in areas of bone that are newly forming at the time when radium is in significant concentration in the circulation (Aub, Evans, Hempleman, and Martland, 1952; Looney and Woodruff, 1953).

Engfeldt *et al.* (1952) demonstrated preferential solubility of the inorganic component of bone in those areas in which radioactive calcium and phosphorus had been recently fixed; *i.e.*, in areas of newly formed bone.

The differences in the proportion of newly formed to long-established bone in animals of different ages are reflected in tracer experiments which involve

the whole animal. Neuman (1953) summarized these differences, noting that "with increasing age the skeleton shows a decreasing reactivity, less exchange, and a lower water content." The extra and intracellular bone water present at any age or any degree of hydration probably exchanges completely as do the salts dissolved in it.

It is obvious that, in reference to the water, inorganic, and organic components (particularly the first two), there are differences in the percentage composition of new and old bone. The question arises as to whether this chemically determined difference is accompanied by a difference in the appearance of the collagen and the inorganic bone crystals in bones of different ages.

What changes in collagen might be expected to take place as bone matures?

Even fixed and dehydrated collagen fibers of *adult* bone in the electron microscope form a "mat" which appears to occupy more than half the volume of the section. Collagen fibers are said to have as much as 75 per cent water by weight when hydrated. In such a hydrated state, one would suppose that they might be larger in diameter and thus occupy even more of the volume of the organic matrix than they appear to do in the electron micrographs of the necessarily dehydrated material.

Fixed and dehydrated collagen fibers from tissue culture as observed by Porter (1951) had increased in diameter after they left the cell periphery where they first took form. This extracellular development appeared to be accomplished by accretion of cell-secreted "building blocks" put into the environment by connective tissue cells. The primary or "skeletal" fiber as it first left the cell periphery had a diameter of only 200 or 300 Å. but increased in diameter to 500 or 600 Å. or whatever the characteristic collagen diameter of the tissue in formation may be. Porter also pointed out that, as the fibers mature, two of the four or five bands noted in each 630 Å. (nominal) subdivision of the fiber became more prominent and eventually formed the doublet banding so characteristic of fully developed collagen fibers.

Electron microscopic and X-ray small angle diffraction studies of tissues rich in reticulin show that reticulin gives the collagen type of pattern, but it can be calculated that the reticulin fiber diameter is less than that of collagen (Bear, 1942, 1944, 1952; Bear, Bolduan and Salo, 1951). It is thought that reticulin fibers may be collagen fibers in an early or "skeletal" phase of their development.

Wyckoff (1952) and Yanamee and Porter (1951), using the electron microscope, reviewed and extended the original observations of Nageotte on the solvation and reconstitution of collagen *in vitro*. Wyckoff thought that his electron micrographs of reconstituting fibers showed peripheral fiber growth. He observed peripheral periodic islands of order extending from the reconstituted fibers into the otherwise disorganized background material.

Using a replica technique for electron microscopic specimen preparation, Huber and Rouiller (1951) found that the average collagen fiber width in the cortex of the femur and tibia in the adult human (52 and 63 years) was 1000 Å. (range: 580 to 1500 Å.), and that bone from the human infant showed an average collagen fiber diameter of 700 Å. (range: 450 to 1200 Å.).

These observations suggested that a dimensional difference might be found in the collagen fibers of newly formed and long-established bone.

What changes might be found in the crystals as bone matures?

Hodge (1949) suggested that the inorganic bone crystals might be very much smaller in newly formed than in stable, long-established bone. He made this suggestion as a possible explanation of the more rapid uptake of radioactive phosphorus by whole subperiosteal and subepiphyseal (*i.e.* subendosteal or trabecular) bone *in vitro* when contrasted to that of compact shaft bone. In discussing this possibility, he also pointed out that Neuman interpreted certain experimental observations as showing an increased rate of recrystallization which might explain the same phenomenon.

Zetterström (1952) pointed out that the crystals in newly formed bone are probably smaller, less perfect, and less stable than those in long-established bone.

Robinson (1952) found that the crystals from subepiphyseal newly formed veal bone were about the same size as those of cortical beef bone. The bone used had been autoclaved at 27 pounds for two to four hours. More recently, undecalcified rat bone was sectioned and compared with comparable bone autoclaved four hours at 27 pounds pressure, plastic imbedded and sectioned (FIGURE 2). Most of the crystals in the sections of autoclaved bone appeared to be larger than the crystals observed in the undecalcified material. Furthermore, there were areas in the sections of the autoclaved material where crystals were absent (FIGURES 3 and 4). Such areas did not appear in the undecalcified material. There was no evidence of tearing of the plastic imbedding material during sectioning of the imbedded autoclaved bone. The autoclaving either increased the size of the smaller crystals, or else the smaller crystals dissolved as the collagen turned to gelatin during autoclaving and trickled out of the bone with the organic material, leaving only the larger crystals behind. Chemical and X-ray diffraction data on bone boiled 24 hours and on the water in which it was boiled, suggest that the crystal size may change and that a *minute* amount (0.03 of 1%*) of inorganic material may be lost in such procedures (Dallemagne, 1950; Robinson, 1953). Finean and Engström (1953), using low angle X-ray scatter diagrams, found no significant evidence of bone *crystal* enlargement after autoclaving. In any event, the *inorganic particles* in sections of "fresh" and comparable autoclaved rat bone appeared significantly larger in the autoclaved than in the "fresh" bone as seen in the electron microscope. The rat bone inorganic particles are about the same size as those seen in newly formed human bone.

In view of the new evidence just presented, Hodge's suggestion that the inorganic particle size might be very much smaller in newly formed subepiphyseal and subperiosteal bone called for investigation by electron microscopy of sections of undecalcified bone of different ages.

When the question arose as to the possibility of observing the submicroscopic changes in the collagen fibers and inorganic crystals of human bone during the maturation process, it seemed best to use subperiosteal bone from the diaphysis

* Robinson, 1953.

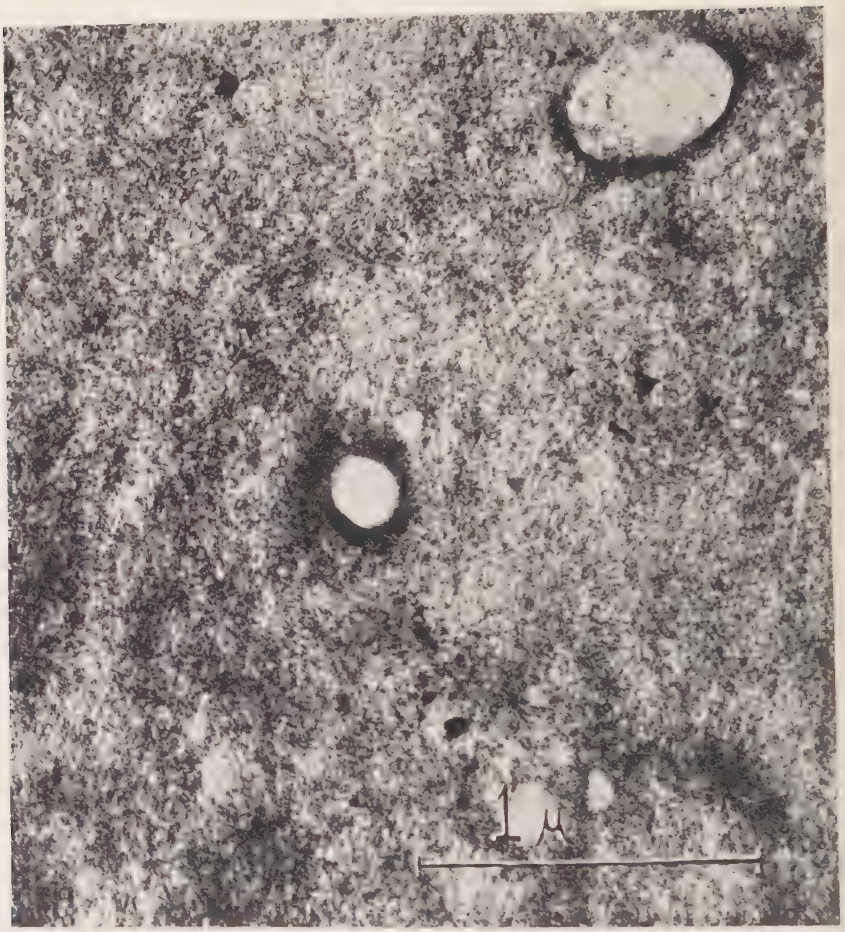


FIGURE 2. Undecalcified rat bone (femoral cortex, section parallel with surface and long axis of the bone). This tissue was fixed in buffered 2 per cent OsO_4 for four hours, dehydrated and imbedded in plastic and sectioned. The very tiny crystal size is apparent, and only in a rare area is there any vague indication of 640 Å. (nominal) periodicity. Most of the objects appear to be less than 100 Å. in size. It will be noted that some areas are more dense than others. Such sections give an electron diffraction pattern characteristic of an apatite. Magnification about ×45,000.

of the human rib. The authors were already familiar with the electron microscopic appearance of bone from the 40-year-old adult in this region (Robinson and Watson, 1952).

Amprino and Engström (1952) found that "The periosteal primary bone reaches a high degree of calcification as soon as it is laid down; in general its calcium content seems not to undergo a detectable increase afterwards." However, there is evidently a period of time during which the matrix of newly formed periosteal bone permits some diffusion and fixation of calcium and phosphorus tracers. This interval may not be as long as in the case of newly formed bone on the surface of a medullary trabecula and is undoubtedly a much shorter

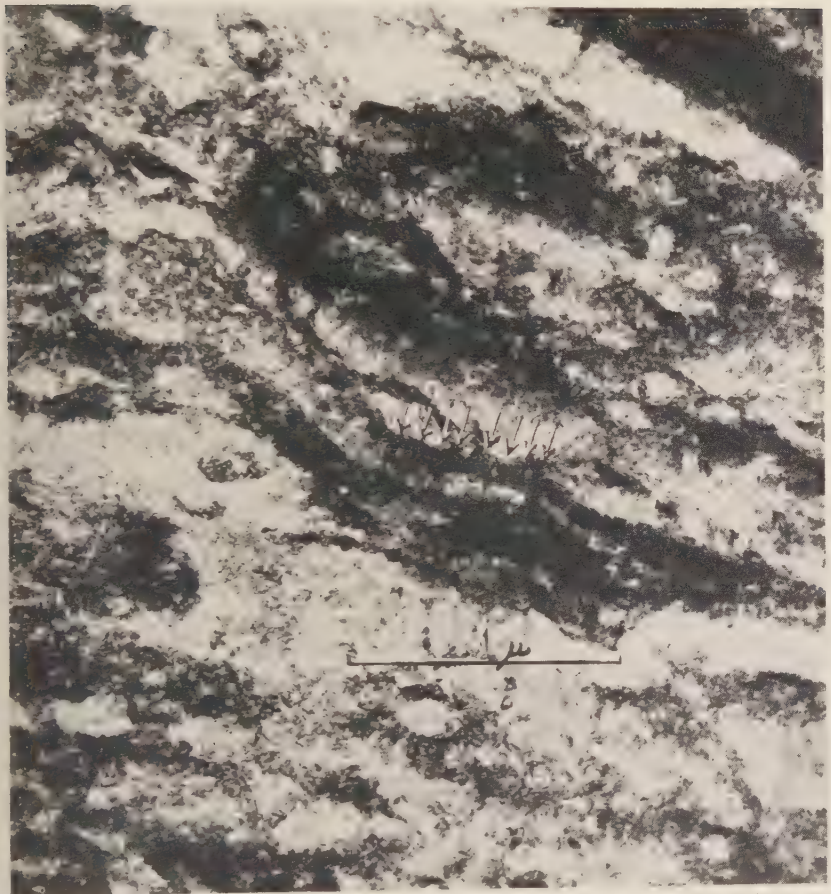


FIGURE 3. Section of the cortex of the femur of a rat. The organic matter of the bone was first removed by autoclaving at 25 pounds for two hours, dehydrated, and embedded in plastic, the material was then sectioned. It will be noted that there are areas of greater and lesser density. In a few areas of the denser regions, such as that marked with the series of arrows, there is a definite suggestion of 640 Å (nominal) periodicity. In this particular section many of the small crystals remain even after the autoclaving and these are particularly well seen between the denser areas of the section. Magnification ×36,000

interval than that obtaining in newly formed osteons. Radioactive phosphorus is picked up by subperiosteal bone for a time after it is laid down, and certainly shows up there in the case of adult rat bones (LeBlond, Wilkinson, Bélanger, and Robichon, 1950). Radium appears in similar regions of human bones (Looney, 1953).

LaCroix (1951) points out that new periosteal bone usually ceases to be deposited on the diaphyses of long human bones at about 24 years. This is about the time when maximum muscle development and remodelling of bones is complete. In old age, the outer circumferential lamellae may become attenuated or even disappear.

The interpretation of the observations which follow are complicated by the

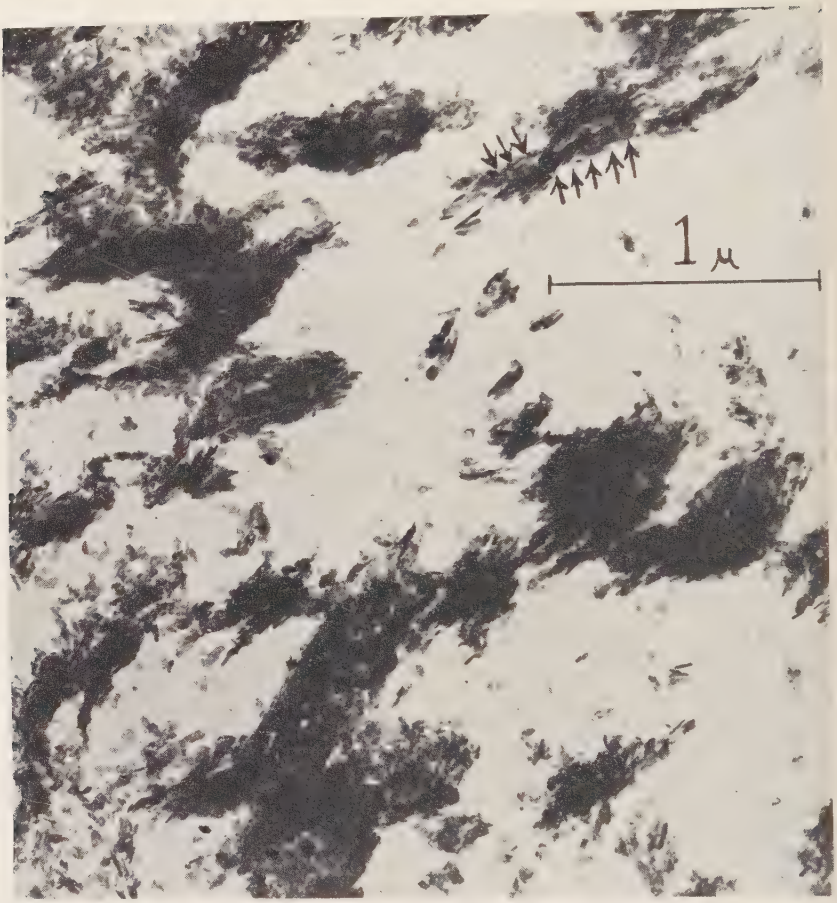


FIGURE 4. The preparation method and bone source was the same as that for FIGURE 3. The magnification is the same. In this section in a few places, as for instance where the row of arrows may be seen, there is a suggestion of a periodic pattern at 640 A. It will be seen that many of the small crystals are not apparent in this section at all, and that in a few places crystals are seen which are relatively large in relation to the general size of the crystal population observed in FIGURE 2. No such crystal free areas were observed in the undecalcified bone sections in which the organic matrix was present, as in FIGURE 2. Magnification ×35,000.

fact that areas of newly formed bone may occur in long-established bone, as well as in bone from infants. However, such areas are much more common in the infant. The sections upon which these observations are based are admittedly spot samples. Some of the sampling in senile bone might pick up an area of newly formed bone, even though the specimen was taken from a place where this possibility seemed least likely. However, the areas described in this work are those which appeared obviously characteristic of the bone of the age under study.

Method

It is believed that the fixation, imbedding, and sectioning techniques (Robinson and Watson, 1952) for bone do not disturb the collagen relationships as do

TABLE 1
BONE CRYSTALS (HYDROXYAPATITE)

Crystal thickness in unit cells @ 8.6 Å. per unit cell	Crystal length and breadth (Å.)	Approximate percentage of total crystal volume occupied by surface layer ½ unit cell thick
1 (8.6)	100 × 75	100
2 (17.2)	100 × 75	68
3 (25.4)	100 × 75	52
4 (34.4)	100 × 75	43
5 (43.0)	100 × 75	38
4 (34.4)	500 × 250	30
6 (51.6)	1200 × 500	19
12 (103.2)	1200 × 500	11

bone fragmentation methods; do not modify the observed crystal size as 27-pound autoclaving appears to do; and do not eradicate the crystals from the collagen matrix as does the acid etching involved in replica techniques of bone specimen preparation for the electron microscope (Rutishauser, 1951).

Thin, longitudinal shavings of human rib cortex taken from the subperiosteal, diaphyseal area on the convex side of the bone were fixed for four hours in 2 per cent OsO_4, buffered at pH 7.3 with veronal-acetate buffer as described by Palade (1952). The bone was washed one-half hour, dehydrated in ethyl alcohol, and imbedded in n-butyl methacrylate.

Sections were cut on a special microtome (Watson, 1953) with a glass knife (Latta and Hartmann, 1950) and floated on to 35 per cent dioxane in water. They were collected on silica-coated collodion-covered grids. The imbedding material was removed by brief exposure to the electron beam near cross-over with the condenser aperture removed (Watson, 1952), except in the case of the material showing a "calcification front" in bone and dentine. In these tissues the imbedding material was not removed.

A possible objection to the use of the high-intensity electron beam to remove the imbedding material from these sections, is that it might result in an increase in crystal size. The pictures of infant bone (FIGURE 5) and rat bone (FIGURE 2) indicate that crystal size is certainly not increased more than 100 Å. by this operation, and that probably there is no observable effect on crystal size.

Decalcification was carried out in about 0.85 normal trisodium versenate* solution buffered at pH 7 with $Na_2 H PO_4 - K H_2 PO_4$. Thin bone shavings in this solution, using constant agitation, required about 24 hours for decalcification. This step was followed by the procedures described above for undecalcified bone.

Rat bone for FIGURES 3 and 4 was autoclaved for four hours at 27 pounds pressure (at a temperature of 134° C.). This bone was then dehydrated in various dilutions of alcohol, imbedded in n-butyl methacrylate and sectioned in the same manner as were the other specimens. The autoclaved bone was much easier to section than was bone in which the collagen was still present.

* Equimolar mixture of disodium versenate and sodium hydroxide. Disodium versenate; *i.e.*, disodium salt of diethylene diamine tetra-acetic acid dihydrate), manufactured in reagent quality by Bersworth Chemical Company, Framingham, Mass.

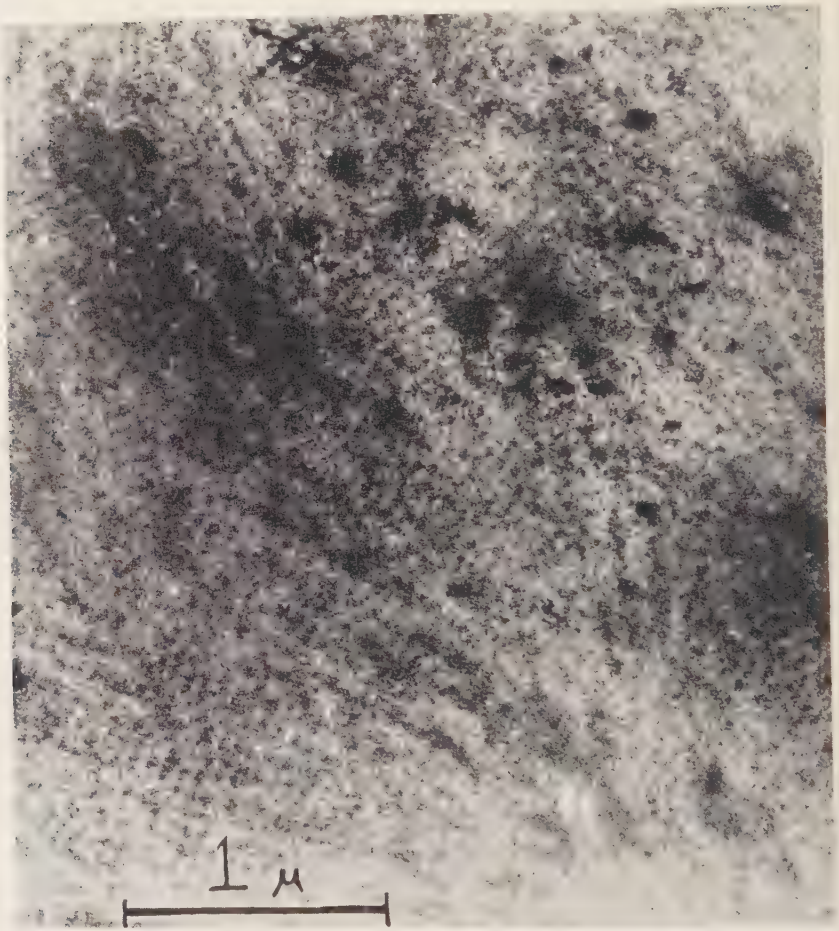


FIGURE 5. Undecalcified human bone, lateral rib cortex from a two-day-old infant. The presence of 640 Å. periodicity is seen throughout the section. However, the inorganic material that is observed is of such small particle size that almost no dimensions can be measured. Rarely, crystals on edge are seen as black streaks which are about 200 Å. in length, but it would seem that they are the largest crystals in the area. Electron diffraction patterns of such sections were rather indistinct but were compatible with an apatite. This material was fixed in 2 per cent OsO_4 for four hours, dehydrated, imbedded in plastic, and sectioned. Magnification ×35,000.

Micrographs were taken with the RCA type EMU-2A electron microscope. An intermediate lens and a standard compensated pole-piece was used in micrographs. In some cases (FIGURES 1, 5, 8, 9, 10, and 17), a 25-μ platinum objective aperture was used.

Results

Important differences were observed in the crystal size in infant, 40-year-old, and the 80-year-old human male subperiosteal rib bone. In general, there is a progressive marked increase in crystal size with age.

In the decalcified bone, significant changes in the collagen fine-structure

FIGURE 6. Undecalcified human bone at a "calcification front." Lateral rib cortex from a two-day-old infant. The presence of 640 Å. periodicity is seen in the uncalcified collagen and in the calcified regions. In the center there is an area showing the small inorganic stippling or "dots" that have a 100 Å. period. At the edge of the fibers in several places, the linear inorganic opacities are seen, that have a length of 200 to 300 Å. This material was fixed in buffered 2 per cent OsO_4 for four hours, dehydrated, imbedded in plastic, and sectioned. The imbedding material was *not* removed. Magnification ×58,000.

the diameter of the fiber, the band pattern, the packing, and the distribution of the collagen have been observed and correlated with age.

Undecalcified Bone

The material seen in the sections of undecalcified bone is almost entirely the inorganic component. Collagen, because of its much lower scattering power, is not apparent (Robinson and Watson, 1952).

(1) *Infant bone.* Close examination of the pictures reveals a fine granular structure which is taken to indicate an average inorganic particle dimension of less than 100 Å. It is not possible, from these pictures, to determine the shape

FIGURE 7. Undecalcified developing hamster dentin, from a "calcification front." The fibers are cut on end or slightly on the oblique. A denser haze is seen at, and in the immediate vicinity of the fibers. Very dense spots are occasionally seen on the edges of the fibers just adjacent to the calcified area. Suddenly, the fibers are obliterated by the calcific densities. Calcific free circular areas are not apparent in the calcified portion. Also at the "calcification front" the fibers on end become electron opaque. The preparation method same as for material in FIGURE 6. Magnification ×58,000.

of these small particles. In some places, an occasional crystal 250 Å. to 300 Å. in length is observed.

In contrast to the undecalcified rat bone (FIGURE 5), the major collagen banding of about 640 Å. is delineated in the infant in many areas by systematic variations in the concentration of inorganic material along the collagen fibers (FIGURE 5). A 100 Å. periodicity is suggested in many places in these electron micrographs of undecalcified infant bone.

FIGURES 6 and 7 are electron micrographs of undecalcified infant bone and undecalcified hamster dentine viewed at the "calcification front." One can see both the uncalcified and the calcified portions in the same field. Immediately preceding the calcified zone, collagen fibers are obviously present. An

FIGURE 8. Decalcified human bone, lateral rib cortex from a two day old infant. This material was decalcified in trisodium versenate, fixed in 2 per cent OsO_4 for four hours, dehydrated, imbedded in plastic and sectioned. The comparatively loose network of fibers is seen, the major periods stand out, although, in some areas, and particularly in the narrower fibers, the major periods are not seen or are seen poorly, where as the interperiod bands which lie about 100 Å. apart are well seen. In general, the wider the fiber the more definite is the 640 Å pattern. Such sections give no apatite pattern with electron diffraction. Magnification ×15,000.

amorphous haze has brought some of the fibers and some of the surrounding interfibril material into extra prominence. There are also, in these regions, longitudinal inorganic opacities with a sort of wavy outline lying parallel to the direction of the adjacent fiber. These are first seen alongside some of the fibers both in sections of bone and dentin. Referring back to the synthetic precipitate of basic calcium phosphate (Robinson and Watson, 1953), such inorganic material appears morphologically similar to the "phase 2" type of precipitate. In "phase 2," the inorganic material consisted of thin sheets of irregular outline and of about 1 or 2 unit cell thickness. This phase preceded "phase 3," which consisted of crystals of definite outline.

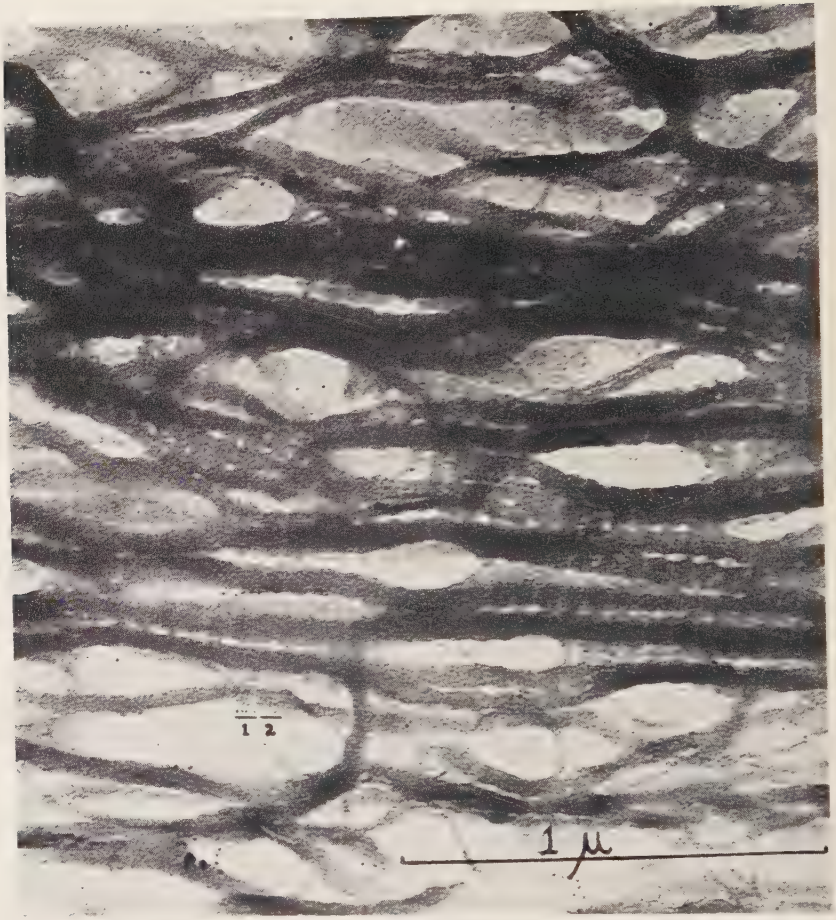


FIGURE 9. The source and preparation of this material was the same as that noted in FIGURE 8. In this preparation it can be determined that the fibers vary in width from 150 Å. to 450 Å. There are about five small bands to one major 640-Å. period, so that there are about 100 to 120 Å. per small period. Measuring across the fibers, as one scrutinizes the fibers carefully, there seems to be a division of these interperiod bands as one proceeds from one side of the fiber to the other, and it would appear as though these bands were not smooth but, rather, that they have a prominence about every 100 Å. as one proceeds from one side of the fiber to the other. The looseness of the collagen packing in sections of infant bone does not modify the observation that when these fibers come close together their major periods seem to fall into phase or come into register and "bridges" at these points of close proximity are noted. Magnification ×55,000.

In some areas, a fine stippling of a density compatible with the inorganic component is observed. It appears to have a periodicity about 100 Å. It also seems to be distributed in relation to the fibers.

(2) *Middle-aged bone.* An electron microscope study of rib bone from a 40-year-old man has previously been reported (Robinson and Watson, 1952). FIGURES 10 and 11 show low and high magnification views of a representative area. The major collagen banding is well marked by the crystals. A large percentage of crystals are found with dimensions of about 400 Å. by 200-300 Å. by 25 to 50Å. Many smaller crystals are also present with a maximum dimension of about 180 Å.

FIGURE 10. Undecalcified human bone, lateral rib cortex from a 40 year-old human male. The collagen fibers are completely masked. The "grain" of the section and information gained from enlargement of this section and from sections of partly decalcified bone such as that in FIGURE 17 and from sections subjected to electron diffraction before and after decalcification establish the following interpretation. Most of the collagen fibers lie parallel to the plane of the section (this section was taken parallel to the surface of the rib and in the long axis of the bone). The inorganic bone crystals form electron dense ribbons or bands which run across the section at right angles to the fiber direction. These crystal ribbons have a width of 400 Å, approximately equal to that of the doublet bands seen in FIGURE 10. These bands are apparently continuous across several underlying collagen fibers. Some areas are much denser than others. It should be noted that, in contrast to FIGURE 5, a definite clear area is present between the bands of inorganic crystals. As can be seen more clearly in FIGURE 11, individual crystals can be observed in many areas. In general, the particle size of the inorganic material is greater in this bone from a middle-aged subject than in bone from the infant. Magnification ×18,500.

(3) *Senile bone.* FIGURE 14 shows part of a section taken from the rib cortex of an 80-year-old man. In general, the major collagen banding was not revealed by a periodic distribution of crystals along the fibers. The crystals are considerably larger than those previously described. They have the characteristic shape of thin plates which has been observed in all bones and enamel crystals which could be resolved. A few of the crystals have lengths of as much as 1500 Å. and can therefore cover two major collagen periods. The

width is about one third the length in the larger crystals. The crystals are distributed with the long crystal axis roughly paralleling the collagen fibers (Robinson and Watson, 1952; Watson and Robinson, 1953).

Decalcified Bone

(1) *Infant bone.* Sections of decalcified human infant rib show rather loosely packed collagen fibers (FIGURE 8). The diameter of most of the fibers ranged between 380 Å. and 530 Å., although a few with diameters as low as 150 A. are present.

The major collagen period ranges from 560 Å. to 620 Å. In some areas, *five* subperiod bands are found (FIGURE 9). In such areas, the major period is marked by a thickening of the fiber. There is little variation in electron opa-

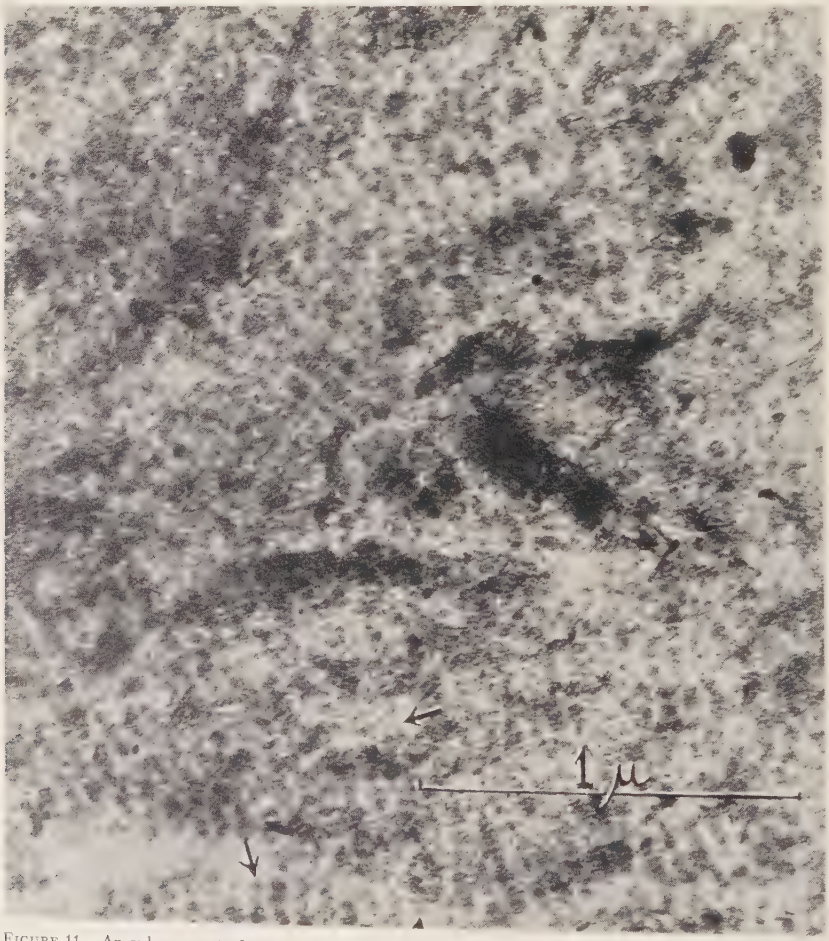


FIGURE 11. An enlargement of part of the section seen in FIGURE 10 to show that in many areas individual crystals can be seen, as for instance where arrows point to one or a group of crystals. Magnification ×49,000.

city along these fibers, except at the small period bands. The resolution in these sections is not adequate to determine whether the *six* small period bands described by Baer (1952) and Schmitt, Hall, and Jakus (1942) in tendon are present in the infant bone. Only five bands were observed in each major period.

In some areas, fibers of the same general dimensions and distribution are seen, but the small-period banding is almost entirely absent. Instead, the doublet banding of the type previously described (Robinson and Watson, 1952) in the larger fibers of 40-year-old rib is present. There is a pronounced increase in electron opacity at the doublet bands in those regions of infant rib where no small interperiod bands are observed.

(2) *Middle-aged bone.* In middle-aged bone, no fibers were found which showed the detailed fine-structure seen in some of the collagen of the infant bone. The doublet banding with a period of about 630 Å. is present exclusively (FIGURES 12 and 13).

FIGURE 12. Decalcified human bone, lateral rib cortex in a 40-year-old male. Decalcified in buffered tri sodium versenate, fixed in 2 per cent OsO_4 for four hours, dehydrated, imbedded in plastic, and sectioned. This bone from a middle-aged individual shows much denser packing of the collagen than that observed in FIGURE 8. On close scrutiny, all of the fibers are about the same size. This uniformity of size can be better observed in FIGURE 13. Magnification ×9,000.

FIGURE 13. Part of FIGURE 12 enlarged. The fibers have a fairly constant width of 800 Å. Small interperiod bands are no longer observed as in the case of the fibers seen in FIGURE 9. Fibers are more closely packed than those in FIGURE 9, but it is still possible to separate them, one from the other, in most places. In some areas, as in the spot marked with the arrow, traces of the small interperiod bands may be observed which existed prior to the dominance of the doublet bands in each major period. ×58,000.

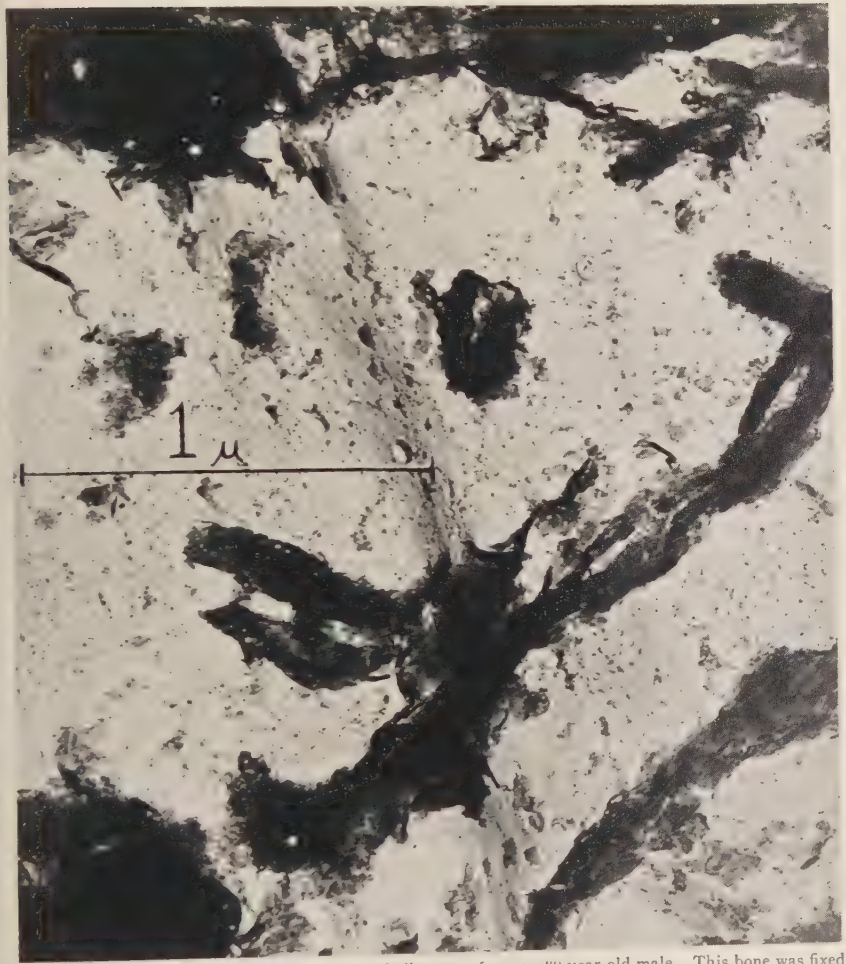


FIGURE 14. Undecalcified human bone, lateral rib cortex from an 80-year-old male. This bone was fixed in 2 per cent OsO_4 for four hours, dehydrated, imbedded in plastic, and sectioned. This material was very difficult to section and tended to fragment. Furthermore, in those areas where fragmentation had not occurred, opacity to the electron beam was so great that no detail could be seen in the electron micrographs. In electron micrographs of this material, it is observed that the crystals are much larger than those observed in the infant or the adult bone. Some of the crystals observed on edge and lying flat against the observation screen have lengths up to 1500 Å., although most of the larger crystals do not exceed 1,200 Å. Small crystals are seen but, in general, the crystal population shows that larger crystals predominate. The long axes of the crystals are seen to lie in the long collagen axis. Magnification ×53,000.

The fibers are more closely packed than in the infant bone, and have a greater diameter, which is fairly uniform, at 800 A.

These observations on adult human bone have been discussed in more detail elsewhere (Robinson and Watson, 1952).

(3) *Senile bone.* With advancing age the packing of the collagen fibers becomes closer and the fiber diameter increases (FIGURES 15 and 16). In senile bone, the fiber diameter ranges from 1000 Å. to 1500 Å. There appears to be

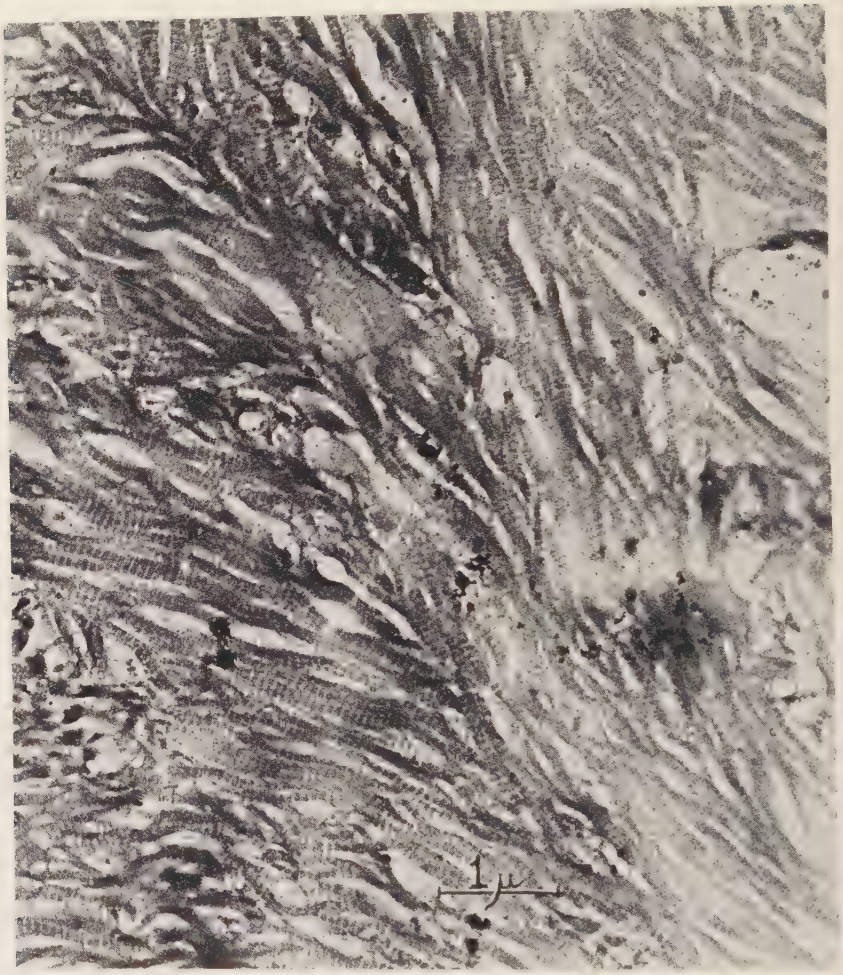


FIGURE 15. Decalcified human bone, lateral rib cortex, 80 year-old male. Decalcified in buffered trisodium versenate, fixed 2 per cent OsO_4 for four hours, dehydrated, imbedded in plastic, and sectioned. The decalcified bone from the senile subject shows very close packing of the collagen fibers. In many places it is difficult to separate one fiber from the other, as though some of the fibers have become practically continuous in their transverse axis with adjacent fibers. Only the doublet banding at the major periods is observed. Magnification ×15,000.

little change in the appearance of the doublet banding from that of the middle-aged bone.

In these sections of senile bone, unlike similarly obtained sections of younger bone, no large areas were found where most of the fibers run parallel.

LaCroix has noted that the outer and inner circumferential lamellae of long bones disappear in senility (LaCroix, 1951). Therefore, thin shavings of the external surface of the cortex of senile human ribs may involve Haversian lamellae, in which the fiber direction changes frequently. It is believed that

FIGURE 16. The source and preparation was identical to that of FIGURE 13. Magnification is greater, being ×44,500. The loss of density of one fiber as to another is characteristic of this collagen of the senile bone. The fiber diameters range between 1000 Å. and 1500 Å

this change of direction explains why "straight fibered" collagen is not often seen in the sections of outer rib cortex of senile bone.

Discussion

There is some indication in the sections of human infant and rat bone of a 100 Å. periodicity in the inorganic phase. This indication, in conjunction with with the small inorganic particle size and the relatively poor demarcation of the 640 Å. period, particularly in the undecalcified rat bone, leads us to suggest that much of the inorganic material initially deposited in such bone is associated with the small period collagen banding. In sections of decalcified human in-

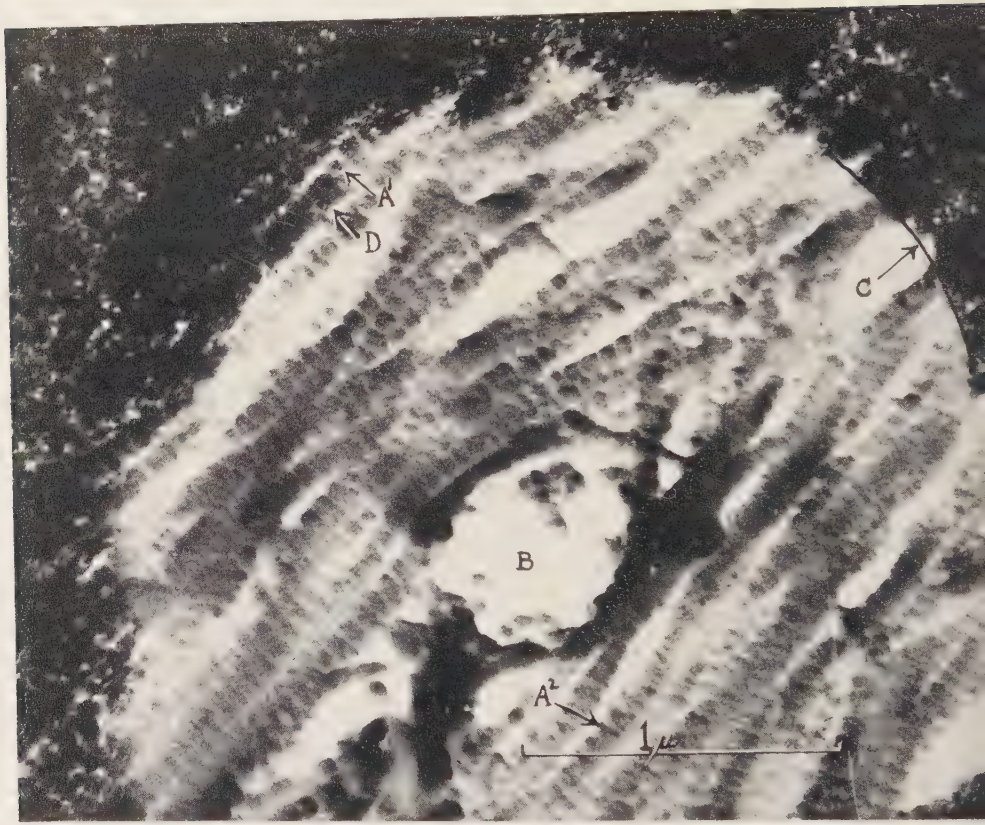


FIGURE 17. Partly decalcified human bone, lateral rib cortex from a 40-year-old male. Partly decalcified in buffe trisodium versenate fixed in 2 per cent OsO_4 for four hours, dehydrated, embedded in plastic, sectioned, and lightly shadow with uranium. Circular areas of noncalcification were seen around canaliculi (B). The edge of the decalcified zone and beginning of the undecalcified matrix is at the arc (C). There is only a very narrow zone about 1000 Å. in width betw completely decalcified and undecalcified bone matrix. In this zone (A^1) and in small areas in other places in the decalcif regions (A^2) some residual inorganic density can be picked up. This density characteristically spans the doublet band a has a width of about 25 to 50 Å. and is separated from the next line of inorganic density by about 25 to 50 Å. These app to be the places along which the inorganic crystals originate on the collagen fibers. These areas of "crystal inception" obviously oriented in the long axis of the collagen fiber. The fibers are clearly seen in the decalcified area and their doub band prominences in relation to the transverse bands of inorganic crystal can be observed (D). Magnification ×41,000.

fant bone, a small 100 Å. to 120 Å. period banding is well defined in some areas of the collagen matrix.

In the "calcification front" in human infant bone and dentin, there appear to be small deposits of inorganic material of indefinite outline, and there also appear to be longer inorganic units. The present impression is that the longer units may be on the periphery of the fibers, while the smaller units may be inside the fibers. The first appearance of inorganic material is definitely observed in the same places where there are collagen fibers. More specifically, the site of appearance seems to be roughly correlated to the bands of these fibers. In FIGURE 15, there are small areas in the decalcified region of adult bone matrix where some residual inorganic density can be seen. This density seems to have a width of about 25 Å. to 50 Å. These linear opacities, about

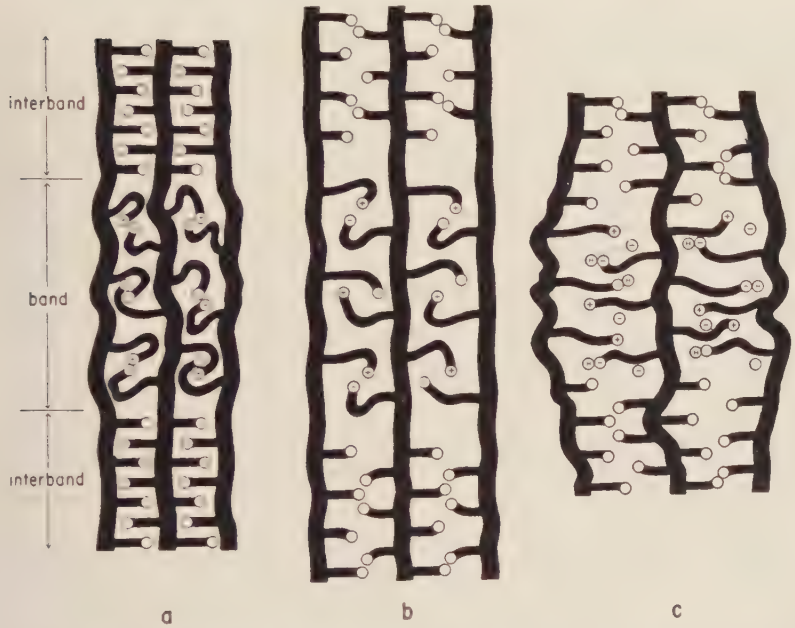


FIG. 32. Diagrammatic representation of the difference between *a*, a dry fibril; *b*, a fibril swelling in water at neutrality; and *c*, the result of acid swelling. Only polar side chains are shown, with open-circled heads representing uncharged side chains, + and − signs designating correspondingly charged heads or ions, and H indicating hydrogen ions. The long charged side chains at bands normally distort the vertical main chain helices from a straight course. Neutral water (not shown) penetrates bands and interbands, separating main chains to an extent limited by hydrogen bonds between polar heads at interbands, and simultaneously more room becomes available for the charged side chains at bands, which now permit straightening of the main chains. Addition of acid discharges the negative side chains by means of hydrogen ions, and the equal number of free negative ions required to remain at the bands produce local osmotic swellings, which contract the structure axially.

FIGURE 18. Reproduced from figure 32 and its caption from Bear (1952), courtesy of Doctor Bear and the Academic Press, New York, N. Y.

200 A. to 300 Å. long, characteristically span the doublet band region of the associated collagen but, in places, they appear to be broken down into smaller units as though the opaque lines were made up of a row of "dots." These opacities apparently represent the foci of crystal inception. They are obviously oriented in lines parallel to the long axis of the collagen fibers.

As maturation proceeds, the collagen fibers grow in diameter and the small period banding seen in the infant disappears (FIGURE 21). A pair of bands (referred to as the "doublet bands") develops at 640 Å. intervals along the fibers. The members of this pair are spaced by about 200 A., and the band width is about 400 Å. (Robinson and Watson, 1952). It is not clear whether these "doublets" represent a new morphological feature or are left behind after the disappearance of three of the small period bands. A "remnant" of one of

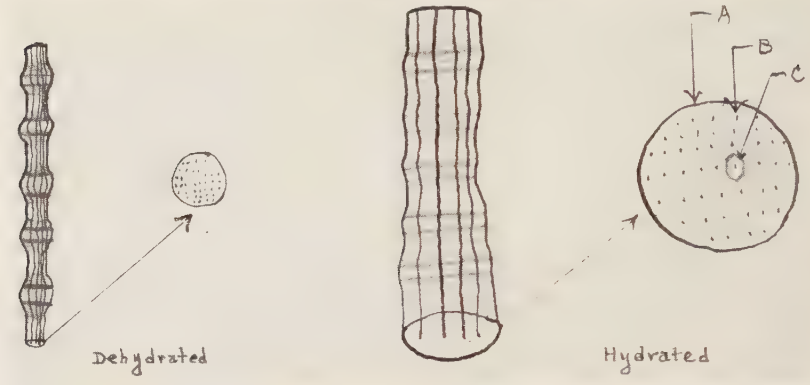


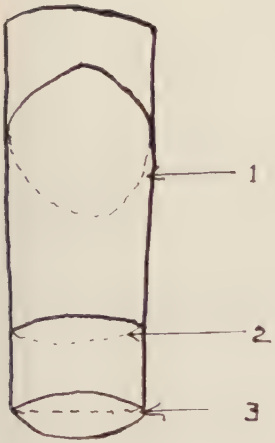
FIGURE 19. A sketch showing the relation of the bands and protofibrils in a collagen fibril as observed in the electron microscope based on Bears model (FIGURE 18): (A) a cross section of the hydrated fibril; (B) a cross section of a protofibril; and (C) the interprotofibrillar area in which water, and probably some cement substance and inorganic component, may be located in bone.

the original five bands has been observed between the pair of bands forming the doublet and, occasionally, traces of one or two bands between one doublet pair and the next have been found on close scrutiny of long-established bone collagen. This finding leads us to the latter view, which is supported by the previously cited observations of Porter (1951): *i.e.*, there is preferential persistence of two of the original five small bands in each major period (FIGURE 21 B).

In bone from the middle-aged subject, in which the collagen "doublet band" exists throughout, the crystals appear to be located over this portion of the fiber, and a relatively clear space between one crystal band and the next is often observed in the undecalcified bone, just as a clear region between one doublet and the next is seen in the collagen of sections of comparable decalcified bone (FIGURE 21 C).

The collagen fibers do not so completely fill the field in young as in older bone, the thickness of the sections being nearly the same. The close packing, obvious in the decalcified senile bone, is presumably due to the change in the fiber diameter. In human subperiosteal rib cortex, it appears gradually to increase from 150 Å. to 400 Å. in infant bone, to a fairly stable 800 Å. in bone from a middle-aged person, and to about 1000 Å. to 1500 Å. in the case of the specimens of senile bone. This observation is in general agreement with the findings of Huber and Roniller (1951).

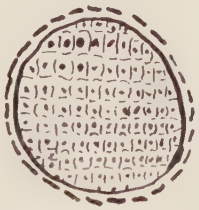
As mentioned at the outset, evidence from various studies of the relative proportion of the organic, inorganic, and water components of bone and enamel suggests that, during maturation of these hard tissues, the percentage of volume occupied by inorganic crystals increases as the water decreases; while the organic volume remains more nearly constant. On the basis of Hunter's observation, a given volume of hard tissue is thought to remain constant during maturation (Dobson, 1948). It is not easy to correlate this concept with the one suggested by the electron micrographs, wherein the fibers which form the bulk of the visualized organic matrix in bone are more slender



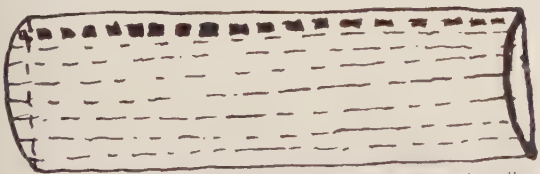
Sections of a fiber.

1 OBlique

2A



2B



3

FIGURE 20. A sketch showing the three possible sections of a collagen fibril: (1) oblique; (2) A and 2B, transverse; and (3) longitudinal. In the case of an oblique or longitudinal section, the crystal density being very much greater than that of collagen will obscure the fiber even though the crystals are all on the periphery of the fiber. Conversely, a true cross section should give a relatively electron lucent region clearly distinguishable in the densely calcified regions if the crystals are all on the periphery (2A). However, if inorganic density permeates the fiber a cross section will also be electron dense. This appeared to be the case in Hamster dentin.

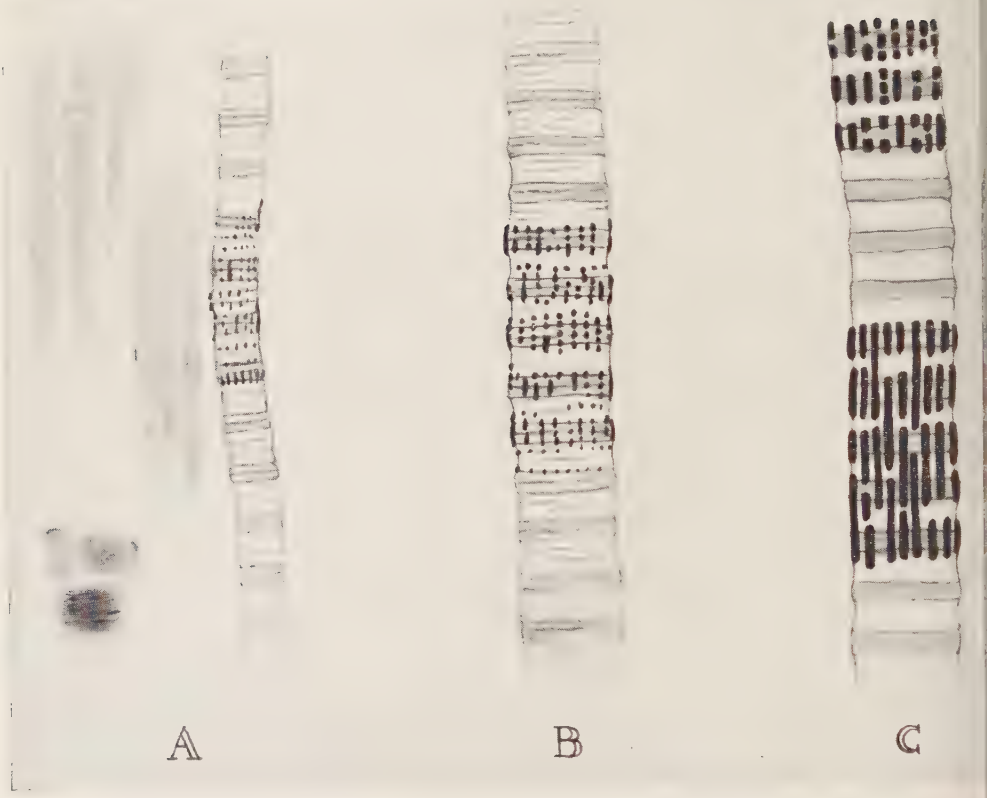


FIGURE 21. This sketch shows what appears to be the course of calcification in bone matrix. (A) First an electron dense haze appears about the fibers and in the adjacent cement substance near the "calcification front." An occasional linear inorganic density appears along the edge of a fiber. In cross section these same densities were observed in developing hamster dentine. The haze vaguely identifies broad bands at the major collagen periods. In some places, small spots of inorganic density with about a 100 Å. periodicity appear in the fiber areas. Suddenly, the fibers are lost and obscured by the calcific densities even though seen in cross section. The decalcified fiber is smaller in diameter, and five bands per major period are seen in some of them, although the doublet band is usually observed. (B) The collagen fibers from more mature bone have many traces of the small 100 Å. period bands, but the doublets are well formed and the inorganic crystals are mostly found in relation to these regions of the fiber as seen in the top portion of (C). (C) The collagen fibers from long-established bone are wider than in (A) or (B) and the crystals are long enough to span two or more major periods, completely obscuring the fiber period.

and less closely packed in new than in long-established bone, and where the noncollagen volume appears to decrease greatly during bone maturation.

The electron microscopic observations, however, do not rule out the possibility that extremely minute units of collagen and other unpolymerized protein and mucopolysaccharide molecules may be present in interfibrillar areas and not yet polymerized, or incorporated into the collagen fibers in newly formed bone. In the case of long-established bone, unpolymerized protein and mucopolysaccharide molecules secreted into the interfibrillar area by the osteoblast may have become incorporated into the collagen during maturation.

The possibility also exists that collagen fibers decrease in density with maturation and expand by internal molecular rearrangement to occupy more vol-

ume, so that when mature fibers are fixed and dehydrated for electron microscopy they do not significantly decrease in diameter. The electron microscope simply shows a much more compact network of larger diameter collagen fibers in longer-established bone. Paralleling the collagen changes in bones of increasing age, an increase in inorganic particle size is observed by electron microscopy.

It should be pointed out that Finean and Engström (1953), using low angle X-ray scattering techniques, came to these conclusions: (1) that bone crystals were rod-shaped; (2) that they had dimensions of 73 Å. by 210 Å.; (3) that their long axes were parallel to the long bone axis; and (4) that their long axes were parallel to the collagen axis.

Our observations with the electron microscope do not support the view that the inorganic particles are rod-shaped. Those particles whose shape can be resolved, appear to be thin plaques in the middle-aged and senile rib. In the senile bone, these plaques reach rather substantial proportions of 1500 Å. by a 500 Å. by perhaps 100 Å. However, few of the particles of infant bone were resolvable in shape at the 50 Å. level. It seems probable that rods as large as 73 Å. by 210 Å. would have been resolved as such, if any large percentage of the inorganic phase had been in this state. Finally, electron microscope studies of synthetic basic calcium phosphates indicated that, under conditions of concentration, temperature, and agitation which were roughly physiological, the only material precipitated was either amorphous or was an apatite with plaque-shaped crystals. Truly rod-shaped crystals were obtained.

We are unable to account critically for this disagreement between our observations and those of Finean and Engström with regard to particle shape. One wonders if plaque-shaped particles whose broad surfaces parallel the collagen axis might produce the same general scattering effect as rod-shaped particles lying parallel to the collagen axis.

Electron diffraction of sections of infant bone gave the characteristic apatite pattern observed in all sections of undecalcified bone examined. The pattern was considerably more diffuse than that obtained from the middle-aged bone. This finding not only confirms the observation of smaller inorganic particle size in the newly formed bone, but it may also indicate that a substantial portion of the inorganic material is *not* crystallized. Such material may be similar to the "phase 1" type of precipitate described in studies of synthetic hydroxyapatite by Watson and Robinson (1953). This early synthetic precipitate did not yield an electron diffraction pattern. It seems likely that a substantial part of the inorganic calcium and phosphate deposited in newly formed bone may be of such a character.

The first appearance of inorganic material is definitely observed in conjunction with collagen fibers and, more specifically, it appears on, and probably in, the 100 Å. bands of newly formed bone and the doublet bands of longer-established bone. Later, crystals gradually fill in the cement substance between the fibers.

What is known of the nature of these transverse bands that appear in collagen fibers? Bear, Bolduan, and Salo (1951), and Bolduan, Salo, and Bear (1951), published what they believed to be a model of a collagen fiber and the effects

observed on this model by hydration, acidification, and tanning procedures. They pointed out that each individual collagen fibril observed by electron microscopy consists of parallel protofibrils or polypeptide chains, 10 Å. to 11 Å. wide, which tend to match their common pattern of chemical features transversely to their axial direction. The models as shown in FIGURE 18 account for the bands (imperfect or distorted locations) and interbands (regions of perfect matching) described by electron optical studies. The sequence of diffraction events accompanying graded introduction of phosphoungstic acid ($H_3P\ W_{13}O_{40}$) into kangaroo tail tendon by these authors indicated that this electron stain entered progressively into the band regions abolishing their contribution to diffraction, but that, up to sorptions as high as 21 per cent, diffraction from perfect interband regions remained. The investigators then attempted specifically to tag side-chain groups on the protofibrils of the collagen fibril which would appear at the imperfect and perfect regions of the fiber; in other words, at the bands and the interbands. It was noted that sodium hexametaphosphate ($6Na \cdot 6H\ PO_3$ or "calgon") combined stoichiometrically with the basic side chains. This combination occurred at the band regions. Bear *et al.* suggested that the residues which would give such a basic reaction were lysine, hydroxylysine, arginine, and histidine. They felt that those which would give an acidic reaction would be aspartic and glutamic acid. The hydroxylic polar types, they felt, would be serine, threonine, tyrosine, and hydroxyproline. Inaccessibility of the hydroxyl polar group to chemical attack would tend to place this group at the resistant interband regions, since it was felt that this group prevented disintegration of the fibers during the various tanning procedures which affected the band regions. They also suggested that over half the residues from collagen were inert and nonpolar, and that these residues might include glycine, alanine, leucine, and proline.

It is suggested that, since the calcification in bone appears to start at the same band areas in the collagen where tanning agents are picked up, and that these two processes, calcification and tanning, may both depend on a similar chemical feature of the band regions: namely, a concentration of acid and basic polar groups.

How might the osteoblast first alter this extracellular matrix to make it a calcium and phosphorus storehouse? Not all collagens respond in the same manner to chemical and physical attack. This difference may not involve the basic fiber as it first appears at the cell border, but it may depend on the type of material that is subsequently attached to it or built into it during its maturation except that the most highly purified collagens show a small but persistent (0.5 per cent ±) hexosamine content. The hexosamines vary. For instance, chondroitin sulfate is found in cartilage and, recently, Neuman and Di Stefano found galactosamine phosphate in calcified rat epiphyseal cartilage and noted its presence chromatographically in embryonic chick cartilage. They believe that it may be present in demineralized compact ox bone. The presence of phosphate in this mucopolysaccharide is the only thing that makes it different from chondroitin sulfate, and the phosphate is attached to the same No. 6 "c" position that sulfate occupies in chondroitin. Once having left the cell surface,

the collagen fiber may be more or less at the mercy of the environment in which it resides, so that, with time and with changing conditions, a fiber unable to calcify at first may later gain this ability. It is tempting to suggest that fibers surrounded by chondroitin sulfate, and perhaps with some chondroitin built into their structure, cannot calcify, while those with galactosamine phosphate and, possibly, citric acid, can calcify.

Up to this time, it has been impossible to make any very convincing statement about the presence or absence of calcium salts inside the fiber. However, we have been fortunate enough to see infant bone and hamster dentin at the "calcification front." First, the fibers are shrouded by an electron opaque haze that makes the major bands stand out. Then, suddenly, the fibers are not only surrounded by inorganic material but this material appears to be present in the fibers themselves. Furthermore, particularly dense round areas in sections of undecalcified bone and dentin have been observed, and it now seems that these are calcified fibers cut at right angles to their long axes. There are no areas in undecalcified, fully calcified, material where electron-lucent, more or less circular areas the size of collagen fibers are seen. Therefore, one must conclude that there is inorganic deposition not only on the periphery of the fiber and between fibers, but also in the fiber (FIGURE 20).

This finding is probably to be expected, if one considers the extracellular bone matrix as a region in which protofibrils of collagen are present in a mass of more or less polymerized cement substance. In some parts, the protofibrils are combined into a fibril with probably a small amount of cement substance between the protofibrils or polypeptide chains. In interfibrillar areas, the space is occupied for practical purposes by cement substance alone. The cement substance contains many things such as citric acid, mucopolysaccharides, glycoproteins, and water.

The actual chemical units in relation to which calcification starts would then appear to be the protofibrils of collagen. In a fiber made up of these protofibrils along with some cement substance, one might expect as high a concentration of the inorganic component as possible within the fibers. The amount of inorganic material in the fiber under normal conditions would be limited to that quantity which would not cause disruption of the collagen fibrils at the interband areas. Crystals are certainly present outside the fibers and they become larger as the bone matures. Whether or not the inorganic component within the fibers becomes crystalline is still questionable (FIGURE 19).

Although Hodge suspected that the crystal size might be much less in newly formed subepiphyseal and subperiosteal bone than in mature cortical bone, he questioned whether difference in crystal size could explain the difference between radioactive phosphorus exchange in whole subperiosteal and subepiphyseal (trabecular) bone of young rabbits and in the cortical shaft bones of adult rabbits. He asked this question: "What kind of a crystal has 50 per cent of its exchangeable atoms on the surface?" (Hodge, 1949). To answer this question, it is important to consider the tabular shape of bone crystals. This shape makes the thickness of the crystal the most important dimension. It is clear that a crystalline plate, one unit cell thick, has all its unit cells exposed to the

surface regardless of its length or breadth. A crystal 20 Å. to 40 Å. thick of the type seen in the middle-aged human bone has approximately 60 per cent to 80 per cent of its unit cells on the surface. Even in crystals of the size seen in senile bone, about 20 per cent to 40 per cent of the unit cells would be on the surface. Thus, a large percentage of the volume of each bone crystal as measured in unit cells is in a surface position, and is in a position to exchange, if conditions outside the crystal are favorable. Quite possibly, *less* than half of the thickness of a surface unit cell is readily available for exchange, or at 37° C. the "surface" may be of *greater* depth (Neuman, 1950). In infant bone, the small particle size markedly increases the ratio of "surface" to "buried" unit cells in the center of the crystal and may play some part in the much greater exchange of bone calcium and phosphorus observed in newly formed bone.

Electron micrographs of undecalcified infant bone show that the size of the inorganic deposits do not usually exceed 100 Å. in length. These deposits are routinely so small that the crystals in such areas could have 50 per cent or more of their calcium and phosphate atoms in their surface layer. Thus, 50 per cent or more of the Ca and P would be available theoretically for exchange at body temperature.

The difference between the uranium exchange of newly formed and longer-established whole bone can be roughly correlated with the surface area difference which is calculated from electron micrographs of crystals in undecalcified infant and senile bone. It is tempting to think that such a correlation with crystal surface area per unit of bone wholly explains the radioisotope exchange difference. However, studies by Amprino (1952, 1953) have shown that bone-seeking isotopes are picked up in much larger quantities (2 to 8 times) by longer-established bone from which the organic matter has been removed by boiling or glycol-ashing. One may not be able to correlate exactly the magnitude of such exchange with comparable data on whole bone because, as has been pointed out previously in this paper, the smaller inorganic particles, such as those found in newly formed bone, may meld and grow at temperatures between 100° C. and 150° C. to a size found in longer-established bone. There are probably changes, accompanying such preparation procedures, in the relative concentration of calcium and phosphate ions on the crystal surfaces, depending on such factors as local hydrogen ion concentration. It should also be pointed out that uranium exchange, in relation to the inorganic crystals, can be postulated only as a surface reaction, while Ca^{45} exchange must be postulated both as a surface and as an isoionic exchange inside the crystal lattice. Nevertheless, although the magnitude of the changes may vary somewhat due to unnatural preparation methods, the general finding seems well confirmed that, with the surrounding organic matrix removed, the crystals in the more calcified and longer-established areas of bone are more readily accessible for isotope exchange, while in the newly formed bone regions, isotope exchange both *in vivo* and *in vitro* is very rapid, whether the organic matrix is present or not (Amprino, 1952d). One must conclude that the organic matrix variations exaggerate the decrease in isotope exchange that one might expect, simply on a basis of crystal growth during bone maturation.

The water content of bone appears to be *inversely* proportional to its age, its fiber diameter, and its crystal size. The water content is proportional (over and above some minimal level of hydration) to its isotope exchange rate. The changes in the water content of bone matrix, the extent of polymerization of the organic matrix, the inorganic crystal size, and the rate of recrystallization, are probably interrelated. Agitation of water solutions, in which synthetic hydroxyapatite crystals were forming, caused smaller crystals to develop. If the Ca P ratio of the solution was insufficient in relation to the Ca P ratio of hydroxyapatite, the original deposit redissolved and no crystals formed (Watson and Robinson, 1953). Agitation mechanically is believed to have caused a lower concentration of ions in solution immediately about the crystals. The result of *in vitro* agitation is thought to be comparable, from the standpoint of the dilution factor, to a more hydrated state of tissue matrix *in vivo*. Therefore, in the more highly hydrated bone matrix of newly formed bone, one might expect smaller crystals and a more rapid recrystallization rate.

It is interesting to consider the histochemical studies on bone from animals shortly after large doses of parathyroid extract. Heller-Steinberg (1951) interpreted her observations as showing a decreased "polymerization" of the "ground substance" in the pericellular regions where there was simultaneously an increased "reactivity" of the inorganic crystalline material. If reactivity of the crystals is characterized by an increased water content of their environment, it is assumed that hydration of the matrix in the reactive areas also increased. Thus, depolymerization of the organic matrix of bone may be associated with crystal "reactivity." One possible mechanism by which more or less water per unit volume could be held in a given volume of matrix would be the presence of more or less polymerized organic molecules per unit volume. During calcification of matrix, polymerization of these organic molecules might "release" water. As this water eventually migrated into the blood stream or was used in hydroxyapatite crystal formation, the dilution factor in the bone matrix would decrease, and larger inorganic crystals might be expected to form on crystal inception points associated with the collagen. Conversely, depolymerization of the cement substance would draw water back into the matrix, and the rate of recrystallization would then increase, while average crystal size would diminish, or the crystal would completely disintegrate. As one reviews this theory, one finds that it is compatible with at least one known fact about resorption of *living* bone, namely: both the organic and inorganic components disappear almost simultaneously, the inorganic possibly preceding the organic by a very small margin.

Although the crystallization nuclei or crystal "motes" are apparently associated with the more electron-dense bands of the collagen fibers, it would appear that the development of a crystal subsequent to its inception, outside of the fiber at least, depends on physicochemical factors affecting the "exposed" crystal surface, such as water content of the matrix, the calcium and phosphorus held by the organic matrix components in its vicinity, and the rate of availability of such inorganic atoms to the surface of the crystal lattice. Only a portion of the elements in the atomic lattice of a crystal can reasonably be expected to be protein-bound. If they were all bound to organic components,

it would not seem possible that an apatite crystal of the thickness found in older bone could be formed. For this reason, no constant ratio of the calcium to nitrogen in bone of all ages seems probable.

Summary

Electron microscopic observations of sections of bone from the outer cortex of human rib, from subjects of different ages, have been made, and progressive changes with age in crystal and collagen morphology noted. With increasing age, the collagen fibers increase in diameter from a minimum, ranging from 150 Å. to 400 Å., to a maximum of about 1500 Å. Some of the infant collagen has a fine-scale banding of about 100 Å., which is displaced in the older subjects to leave a doublet banding recurring about every 640 A.

The bone crystals increase in size from an unresolvable particle of less than 50 Å. in the infant to crystals 1500 Å. by 500 A. by about 100 A. in the senile subject. Much of the infant inorganic component may be noncrystalline.

The influence of the organic matrix on hydration, crystal turnover, initial lattice deposition, and crystal size is considered.

Conclusions

(1) The collagen fibers of bone, as observed after versene decalcification, osmic acid fixation, and plastic imbedding, increase in size as the bone tissue matures.

(2) The five smaller band regions in each major period of collagen fibers, observed in newly formed bone, give way to two-band regions in more mature bone. The two bands are described as a "doublet."

(3) The inorganic component of bone is deposited in the band regions of the collagen.

(4) At a "calcification front," very small inorganic deposits about 20 A. to 50 Å. are seen. It is our impression that these deposits are associated with the small period bands. Bits of other inorganic material, of a more obviously crystalline form, are observed. They are about 200 A. to 300 A. long and about 20 Å. thick. They parallel the long collagen axis and appear to be definitely associated with the doublet band areas that recur about every 640 Å. along the fiber axis.

(5) In general, the smaller crystal and collagen sizes are associated with a more hydrated and less fully calcified matrix.

(6) The less distinct electron diffraction pattern obtained on recently calcified bone matrix suggests that the crystals are not only smaller, but that some of the observed inorganic components in newly formed bone may not be in a crystalline form.

(7) It is pointed out that both tanning agents and the bone crystals are associated with the band regions of collagen.

(8) It seems likely that some of the inorganic component is inside the collagen fibers at the band regions as well as on the periphery of the fibers and in the interfibrillar areas. Electron micrographs suggest this position for some of the inorganic material in the fibers.

(9) The smaller crystal size in newly formed bone is considered in relation

to the larger isotope exchange which occurs *in vivo* and *in vitro* in newly formed bone. The crystal shape is also considered in regard to isotope exchange. However, the degree of hydration and physical state of the organic matrix surrounding the crystals, rather than crystalize, is believed to be the major factor affecting isotope exchange with the crystals.

(10) Attention is called to a possible relationship between the degree of polymerization of the bone matrix and the hydration of that matrix and the crystal size and recrystallization rate. The work of Heller-Steinberg is discussed in relation to the polymerization of the organic matrix cement substance and bone crystal "reactivity." This latter work suggests that the parathyroid hormone is associated with this mechanism.

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FRONTISPIECE TO "FORMATION OF BONES AND TEETH AS VISUALIZED BY RADIOAUTOGRAPHY"

FIGURE 1 Frontispiece. Diagram of the first cusp of the first molar of the rat a few days before eruption. On the right-hand side, the dentinal organ displays from inside outwards pulp, layer of odontoblasts, predentin, and dentin. On the left, the enamel organ shows stratum intermedium, ameloblasts and the four zones of enamel, that is, pre-enamel, young, transitional, and mature enamel.

FORMATION OF BONES AND TEETH AS VISUALIZED BY RADIOAUTOGRAPHY*

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Shortly after the introduction of radioactive elements as a tool in biology, Hevesy and others realized that the mineral component of hard tissues not only insured their mechanical strength but also played a role in the regulation of the mineral metabolism of the whole body. In fact, mineral ions pass continuously into and out of the hard tissues. However, little is known of the factors influencing this dynamic behavior, or of the proportion of the minerals entering hard tissues which is used for growth. One of the major difficulties has been that chemical investigations generally dealt with a whole bone or tooth, whereas the pronounced differences in the histological structure of their various parts suggest equally pronounced differences in mineral behavior.

A detailed analysis of the displacements of minerals within hard tissues can be achieved by radioautography (also called autoradiography), a method by which the radioelements injected into an animal can be located in a tissue section by virtue of the effect of their emission on a juxtaposed photographic emulsion. Radioautographic studies of bone were pioneered by Lomholt (1930) and Behrens and Baumann (1933), but they used lead isotopes (radium D and thorium B) which are unphysiological, if not toxic, and detected them by "contact radioautography," a technique permitting only a crude localization of the radioelements in tissues.†

In order to study the normal growth of hard tissues, it was necessary: (1) to have alpha- or beta-emitting isotopes of physiological elements; (2) to obtain them sufficiently pure to be administered in minute doses which would not upset the normal metabolism; (3) to adopt a radioautographic technique suitable for a precise localization of the radioelements under study. These conditions could be fulfilled since developments in nuclear research led to the production of radiophosphorus and radiocalcium with a high specific activity. It was then possible to inject an amount or weight of material sufficiently small not to produce a significant increase in the amount present in the circulation. The labelled substances could then act as true "tracers" of the normal metabolism of hard tissues. The conclusions reached could, therefore, be used to draw a reliable picture of the sequence of events occurring under normal conditions.

Meanwhile, the introduction of an "integrated method" of radioautography, by which an intimate contact between section and emulsion is provided (L. F. Bélanger and C. P. Leblond, 1946), improved both the resolution and ease of handling of radioautographs. While various forms of this method were described (see review by J. Gross *et al.*, 1950), the fluid-coating and inverted techniques have generally been used in recent work with hard tissues.

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† Radioautographic studies of the tooth with various isotopes were summarized by Bartelstone (1954).

This work consisted initially of an examination of radioautographs of teeth and bones at various time intervals after injection to growing animals of P32-phosphate or Ca45 salts. It was thus hoped to detect the sites and modes of deposition of the mineral *crystals*. Later, it was found that components of the *matrix* of these hard tissues could also be visualized by radioautography, using C14-bicarbonate or S35-sulfate. The main purpose of the present report is to compare the behavior of these four elements in teeth and bones and thus to draw tentative conclusions as to mode of formation and growth of these hard tissues.

For the purposes of the present report, the radioautographic findings on hard tissue formation, growth, and development were subdivided into studies on dentin, enamel, and bone. The results obtained on dentin will be described at greater length than those of the other hard tissues. The reasons for doing this are that (1) development of dentin is less complex and more readily analyzed than that of either enamel or bone; and (2) mineral deposition in dentin as shown by radioautography consists of processes similar to those found in bone and enamel.

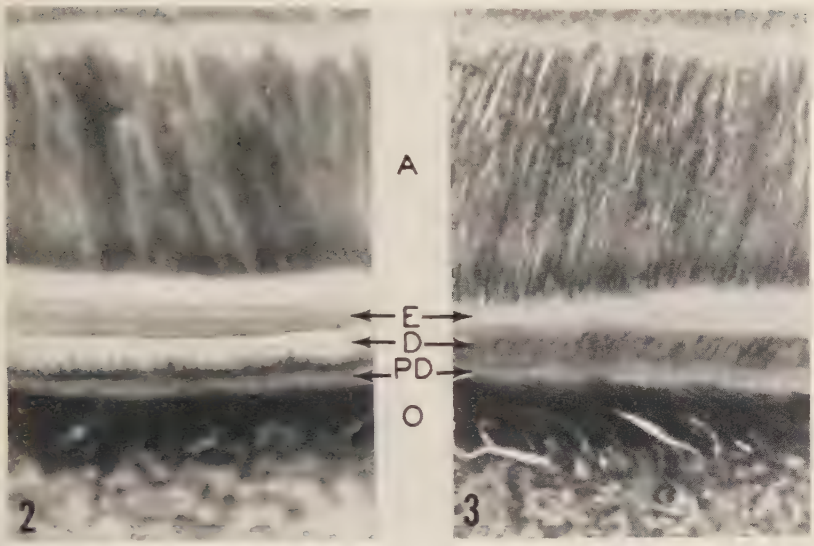
Dentin

Dentin arises out of the activity of the *dentinal organ* (FIGURE 1), which comprises (1) the outermost cells of the pulp, or *odontoblasts*; (2) a layer of noncalcified tissue, the *predentin*; and (3) at some distance from the odontoblasts, a layer of calcified tissue, the *dentin proper*. In decalcified sections (FIGURE 3), the distinction between predentin and dentin, or more precisely between predentinal and dentinal matrix, can clearly be shown by two techniques which stain polysaccharides: periodic acid-Schiff and metachromasia. With both, predentin stains weakly and dentin intensely, so that the line of demarcation between the two tissues is sharp. When the sections have not been decalcified (FIGURE 2), the metachromasia of the dentinal matrix is obscured, but a narrow reactive line is present at the predentino-dentinal junction.

Formation of the matrix of dentin. Formation of dentinal matrix has been studied by several groups of investigators using biologically important isotopic elements, namely, radioactive carbon (C14) and radioactive sulfur (S35). The initial radioautographic demonstration of isotope uptake in dentinal matrix (J. Verne *et al.*, 1952a) indicated that, after administration of *sulfur 35* as sulfate, there was a deposition of organically-bound sulfur 35 in both predentin and dentin.*

Bélanger (1953, 1954b) analyzed the phenomenon in detail. Immediately after injection of S35-sulfate, some radioactivity appeared in dentin, most of which was diffusely distributed throughout its thickness (FIGURE 4). This sulfur could be removed by decalcification and, therefore, was tied up with mineral elements. If the animal was allowed to live for some time after the injection of S35, the diffuse reaction was no longer present (as may be seen in animals sacrificed two days later; FIGURE 5). Presumably, when the blood level of radiosulfate is high, this element is deposited on the surface of the dentin

* Previous radioautographic studies of dentin dealing with substances that were probably not associated with the mineral component of the tooth were carried out using sodium-24 ion (H. Berggren, 1946) and S35-cystine (G. B. Wislocki and R. F. Sognnaes, 1950).



FIGURES 2 and 3. Metachromatic areas in undecalcified (FIGURE 2) and decalcified (FIGURE 3) sections of the root of a rat incisor stained with toluidine blue. ×360

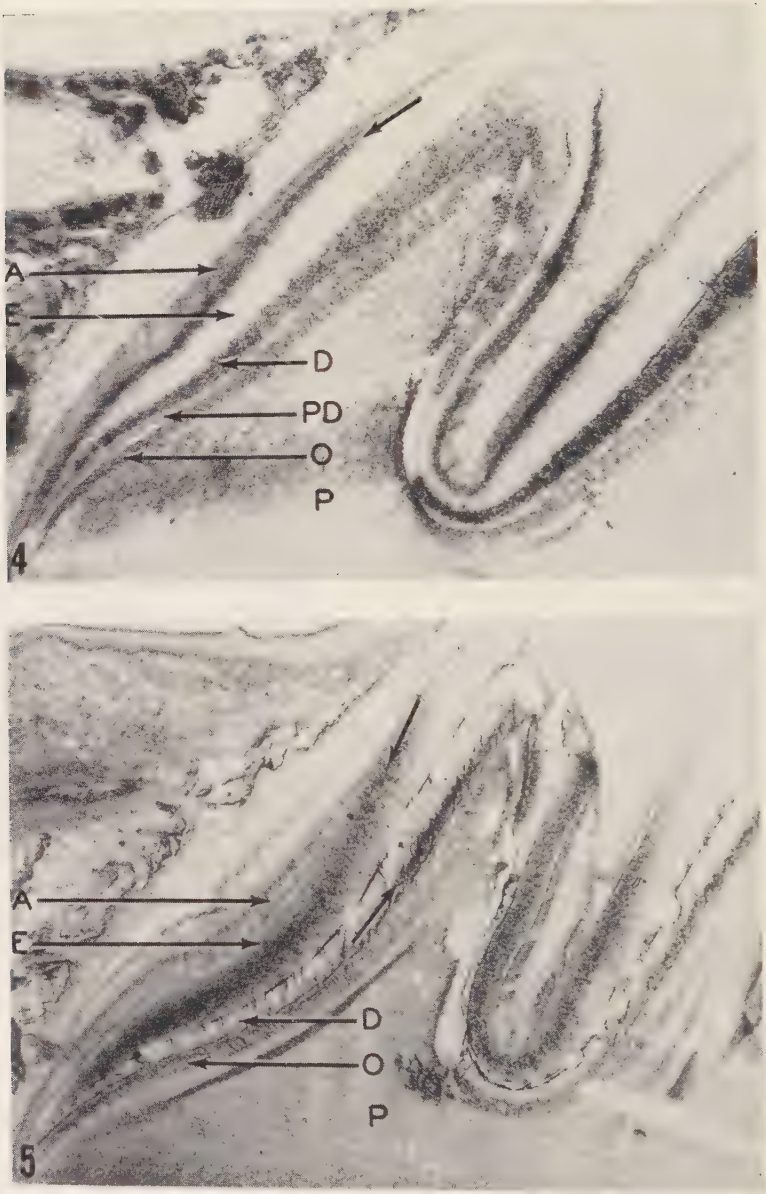
The undecalcified section (FIGURE 2) shows that the predentin stains a light purple while a deep purple color is visible at the limit between predentin and dentin. Dentin does not stain except for a slight reaction associated with dentinal tubules.

The decalcified section (FIGURE 3) also shows a light purple stain of predentin, but dentin stains intensely. None of the other materials stained in this section showed the purple color characteristic of toluidine blue metachromasia.)

crystals by a process of adsorption or by exchange with phosphate ions, while the reverse of these phenomena occurs when the blood level of radiosulfate drops.

Some of the radioautographic reactions observed after S35 injection were not removed by decalcification and therefore were due to the inclusion of labelled material in the matrix (L. F. Bélanger, 1953, 1954b). At the 2-hour interval, the radiosulfur of the matrix was arranged as a band of reactive material lying at the junction between predentin and dentin (FIGURE 6, ascending arrow), while a slight reaction arranged as a decreasing gradient therefrom extended throughout predentin. As in the other cases to be reported below, the main band was, in fact, the cross section of a layer of newly formed labelled matrical material. At later intervals after S35 injection, the band was found within the dentin at a progressively increasing distance from the odontoblasts (FIGURES 5 and 7, ascending arrows) as a result of the continuous accretion of new layers of dentinal materials which, after the level of circulating isotope had dropped, were no longer significantly labelled. In addition, there seemed to be a progressive decrease in the intensity of the reaction band with time (compare the intensity of the bands in FIGURES 5 and 7, both exposed for the same length of time).

When *carbon 14* was administered to newborn rats as labelled sodium bicarbonate, the isotope was observed first in the predentin (FIGURE 8) and later in the dentin of molars and incisors (R. C. Greulich and C. P. Leblond, 1953). It was suggested in this work that the radioautographic reactions were not due



FIGURES 4 and 5. Radioautograph of the first cusp of the first molar in rats injected with S35-sulfate at the age of eight days and sacrificed *two hours* (FIGURE 4) or *two days* (FIGURE 5) after injection. Undecalcified sections; inverted technique, basic fuchsin stain; retouched; from L. E. Bélanger, 1954a.) At two hours after injection (FIGURE 4), the pulp (P) shows a diffuse reaction which is maximal in an area bridging the entrance to the pulp cavity. The predentin (PD) shows little reactivity. The dentin (D) shows a diffuse reaction extending throughout (in decalcified sections, the diffuse reaction was not observed, but the bandlike reaction illustrated in FIGURE 6 was visible, this reaction is due to S35 incorporated in the matrix). The enamel organ shows an intense band of reaction which appears to be at the limit between the tallest ameloblasts (A) and the enamel (E). This reaction, indicated by the descending arrow, is located in pre-enamel. In the upper left corner, bone trabecules exhibit a diffuse reactivity.

At two days after injection (FIGURE 5), the main changes are as follows: the diffuse reaction of the dentin is no longer present; the bandlike reaction due to the matrix S35 persists (ascending arrow); the enamel reaction (descending arrow), which now spreads out throughout the young and transitional enamel is wider than at the earlier interval (FIGURE 4); and the reaction of the bone tissue is almost completely gone.

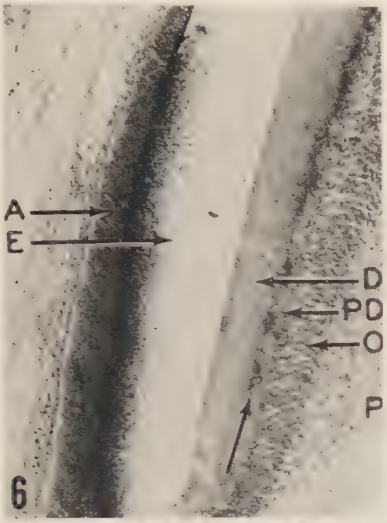


FIGURE 6 Radioautograph of the anterior wall of the first cusp of first molar in a rat injected with S35 sulfate at the age of eight days and sacrificed *two hours* later. (Decalcified section; inverted technique; basic fuchsin stain; from L. F. Bélanger, 1954a.) ×108

There is a moderate reaction of the predentin (PD) and a more intense reaction at the limit between predentin and dentin (ascending arrow). The intense reaction of the pre-enamel is indicated by an arrow pointing downwards.

to the incorporation of carbon into the mineral, but rather into the matrical elements of dentin, presumably as high molecular weight organic compounds. Eventually, the presence of C14 within the matrix was proved conclusively (R. C. Greulich, 1953a, b).

More recently, the entry of radiocarbon into the matrix was systematically examined in a series of young rats killed at intervals of from 1 to 15 days after the administration of C14-bicarbonate (C. P. Leblond *et al.*, 1953; R. C. Greulich and C. P. Leblond, 1954). The C14 utilized in the synthesis of organic matrix by the dentinal organ appeared in radioautographic preparations in the form of a band which, with time, seemed to be farther and farther from the odontoblasts, but remained at approximately the same distance from the dentino-enamel junction (FIGURES 9–12).

The foregoing findings demonstrate conclusively that both radiocarbon and radiosulfur may participate in the synthesis of organic components of dentinal matrix. On the basis of its amino acid composition, the matrix is known to be composed mainly of collagen (W. C. Hess, C. Lee and B. A. Neidig, 1952b). In addition, two carbohydrate-containing substances were extracted: an acid mucopolysaccharide, identified as chondroitin sulfate (W. C. Hess and C. Lee, 1952a), and a carbohydrate-protein complex containing galactose, mannose, and fucose (unpublished work with Kumamoto and Glegg). The acid mucopolysaccharide is held responsible for the metachromasia and the carbohydrate-protein complex for the periodic acid-Schiff staining of the matrix (unpublished). Accordingly, the staining differences between dentin and predentin (FIGURE 3) indicate that the two types of carbohydrates are much more abundant in dentin than predentin.

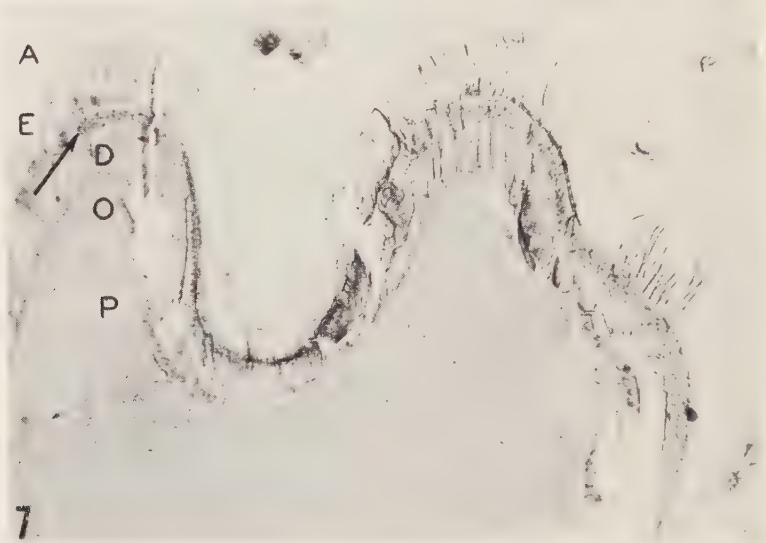


FIGURE 7. Radioautograph of the first molar (cut to the side of the apex) in a rat injected with S35-sulfate at the age of eight days and sacrificed *six days* later (undecalcified section; inverted technique; basic fuchsin stain). ×52

The reactive band (arrow) of the dentinal matrix is visible within the dentin, although fainter than at early intervals. The reaction of enamel is no longer visible at this time (the decrease in these reactions suggests the existence of a turnover of the S35-labelled material).

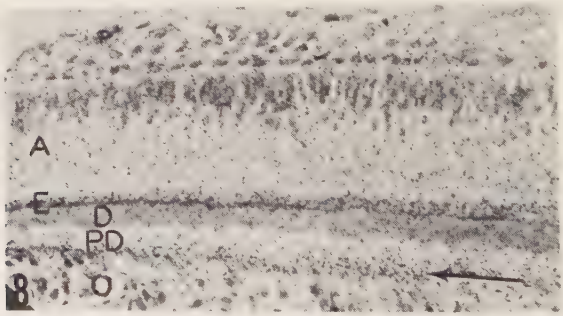
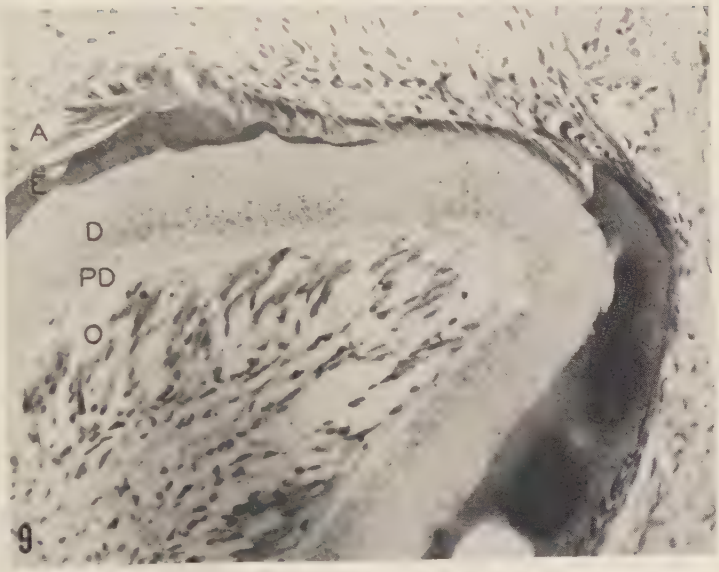
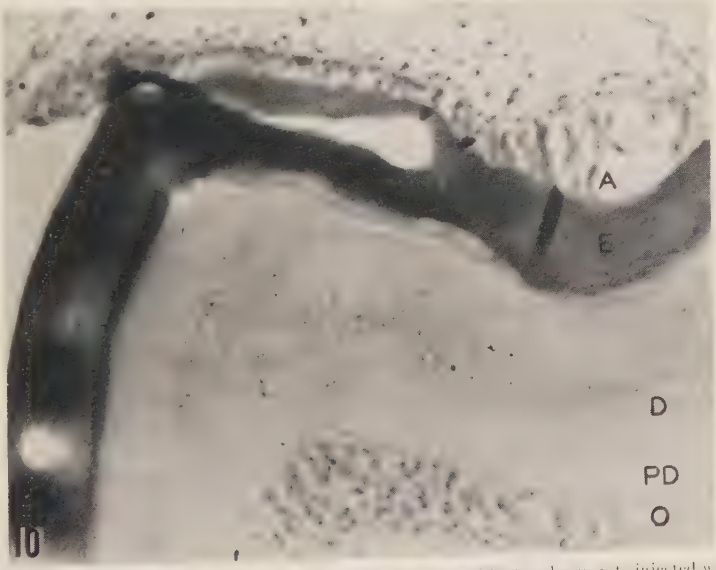


FIGURE 8. Radioautograph of a portion of incisor root in a rat injected with C14-bicarbonate (40 μc.) at birth and sacrificed four hours later (decalcified section; coated technique; H. and E. stain; from R. C. Greulich and C. P. Leblond, 1953). ×180

The autographic reaction (arrow) predominates in an area of predentin adjacent to the tips of the odontoblasts (O). The darker staining dentin (D) is unreactive. Pre-enamel (E) exhibits a precise but slight reaction.

It is known that S35-sulfate participates in the formation of sulfated mucopolysaccharides (H. Borström and E. Odeblad, 1953; L. F. Bélanger, 1954b). The last-named author observed that sections of dentin treated with hyaluronidase before radioautography showed no reaction — a fact indicating that the S35-labelled component of the matrix was hydrolyzed by the enzyme and may, therefore, be presumed to be chondroitin sulfate. The initial predominance of the radioautographic reaction at the predentino-dentinal junction (where an intense metachromasia may also be seen, FIGURE 2) indicates that the bulk of

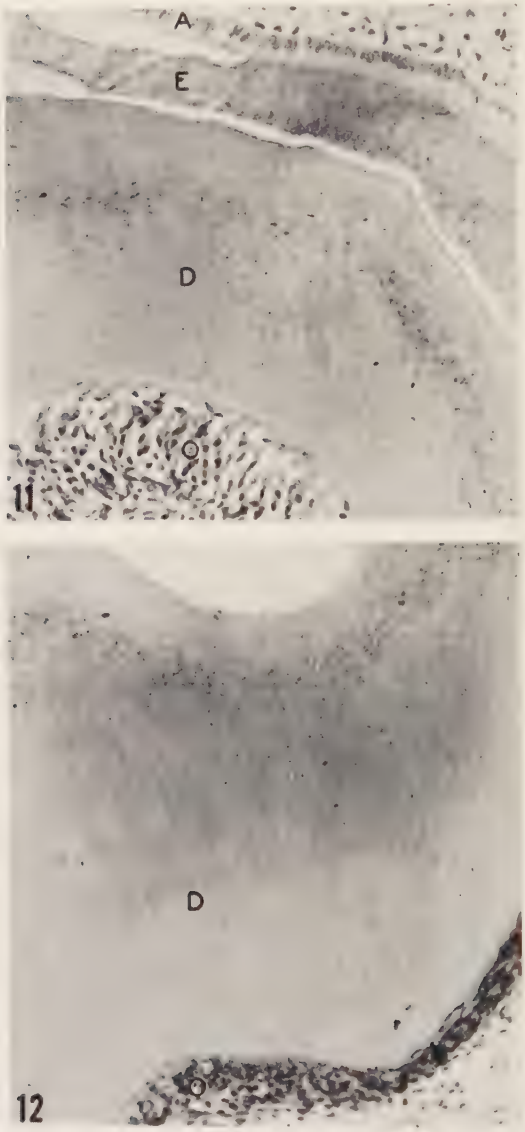




FIGURES 9 and 10. Radioautographs of the tip of the first cusp of first molar in rats injected with C14-bicarbonate when three days old and sacrificed *one day* (FIGURE 9) or *three days* (FIGURE 10) later; decalcified section; coated technique; H and E stain; from R. C. Greulich and C. P. Leblond, 1954. ×180.
The predentin (PD) is unreactive, but a band of reaction is visible in the dentin (D), close to the predentin (FIGURE 9, or deep in the dentin (FIGURE 10). In both cases the distance between the band of reaction and the dentino-enamel junction is the same.

the chondroitin sulfate of dentin is synthesized, or at least sulfated, at the very zone where predentin transforms into dentin.

The chemical form of the C14 incorporated into predentin (FIGURE 8) is not known. Since histochemical tests suggest that collagen is abundant and



FIGURES 11 and 12. Radioautographs similar to FIGURES 9 and 10 but in rats sacrificed *6 days* (FIGURE 11) or *15 days* (FIGURE 12) after C14-bicarbonate administration. ×180
The bands of reaction are seen to be farther and farther from the odontoblast layer but remain at approximately the same distance from the dentino-enamel junction. There is no decrease in the intensity of the reaction with time (presumably, there is no turnover of the C14-labelled material).

carbohydrates are scarce in predentin, it is believed that the C14 initially found in predentin is in the form of collagen.

In conclusion, it is suggested that the dentinal matrix arises in two steps: (1) *Formation of predentin*, visualized by the appearance of C14-labelled

material (collagen?) on the inner surface of the predentin in immediate contact with the odontoblasts (FIGURE 8). With time, the accretion of newer (unlabelled) material displaces the radioactive layer farther and farther from the odontoblastic layer (FIGURES 9 12). About 24 hours are required for the C14-labelled material to ascend through the area of predentin to the point where transformation into dentin takes place.

(2) *Formation of dentinal matrix* by transformation of predentin at the predentino-dentinal junction. This step is not associated with any apparent change in the C14-labelled material; and, therefore, components of the predentinal matrix persist as part of the dentinal matrix. Furthermore, the appearance of S35-labelled material at and near the junction indicates that a sulfated mucopolysaccharide has been added in this area. In fact, the transformation of predentinal into dentinal matrix may be due mainly to the incorporation of this type of carbohydrate (in addition, the much more intense staining of dentin than predentin with the periodic acid-Schiff technique suggests the possibility that a carbohydrate-protein complex is also added at the junction).

With time, the S35-labelled material deposited at the junction, as well as the C14-labelled material from the predentinal area, are found lying more and more deeply in the substance of the dentin as a result of the continuous accretion of new, unlabelled components of the matrix.

Formation of dentinal mineral. Since the minerals of dentin have previously been shown to consist chiefly of phosphate or carbonate salts of calcium, this section will be concerned with the experimental radioautographic study of radioactive precursors for the components of these salts: P32-phosphate, C14-bicarbonate, and calcium-45 ions. The deposition of other mineral elements in teeth and bones has been reviewed elsewhere (J. Gross and C. P. Leblond, 1948; J. Gross *et al.*, 1950). At the outset, it should be pointed out that the available experimental evidence indicates that the general pattern of deposition and subsequent fate of radioactive calcium is similar to that of radioactive phosphorus. For this reason, the main body of the presentation will concern itself with data obtained from studies of the radioactive phosphorus distribution in dentinal minerals.

In young rats, *radiocarbon* administered as bicarbonate ion played a negligible role in the formation of dentinal minerals (R. C. Greulich, 1953b); whereas, in older rats, C14-bicarbonate was incorporated into these minerals (W. Bloom *et al.*, 1947; W. D. Armstrong *et al.*, 1948). It is possible that young animals fed on maternal milk are supplied calcium and phosphate ions in a ratio suitable for formation of dentinal salts, while old animals are likely to be fed a diet unbalanced in this regard and, as a consequence, carbonate ions may be deposited. The difference in carbonate uptake between young and old animals deserves further investigation.

The deposition of *radioactive phosphorus* in newborn mice was shown to occur throughout teeth and bones (L. F. Bélanger and C. P. Leblond, 1946). It was later stated that in newborn rats predentin did not, while dentin did, take up radioactive phosphate (W. L. Percival and C. P. Leblond, 1948). These authors reported a positive correlation between the distribution of P32 and of

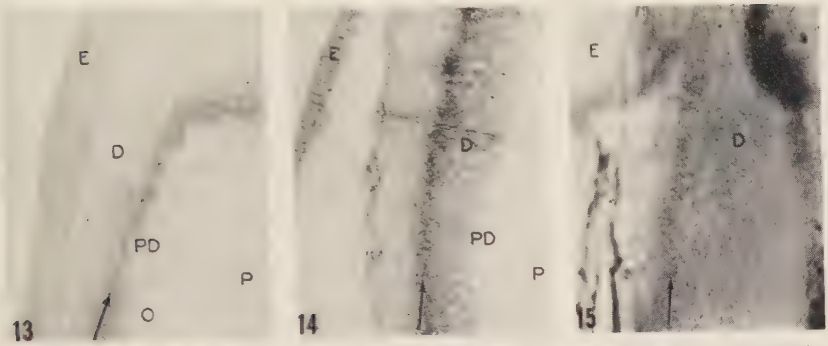
histochemically demonstrable phosphate salts. The conclusion was then reached that radiophosphorus distributed itself like preformed phosphate in bones and teeth of newborn rats.

Today, it is still found that the dentinal tissue of newborn rats accumulates phosphate throughout its entire extent, although improvements in the radioautographic technique have revealed differences in intensity within the various portions of the dentin, particularly in incisor teeth. This difference was shown in an investigation of the deposition of radiophosphorus in growing molar teeth of young hamsters (L. F. Bélanger and C. P. Leblond, 1950). In this study, the animals were injected with radioactive phosphate when they were four days of age and were sacrificed subsequently at intervals ranging from one hour to four days after injection. Two types of autographic reaction were visualized, *diffuse* reactions extending throughout dentin and enamel, and *localized* reactions restricted to definite parts of these structures. The localized reaction persisted as a "band" which initially was observed in dentin at a short distance from the odontoblasts. Examination of the animals killed at later intervals revealed that this band was then displaced farther and farther from the odontoblasts. The conclusion was drawn that dentin in these young animals grew as a result of the continuous accretion of layers of insoluble phosphate on its inner surface close to the odontoblasts.

In a more detailed study of phosphate metabolism in dentin as visualized by radioautographic techniques, the teeth of hamsters and rats injected at the age of four days were examined up to 10 days after injection (L. F. Bélanger, 1952). The existence of both the diffuse, or exchange-type, and the bandlike, or growth-type, of radioautographic reaction was confirmed. The latter reaction appeared in the vicinity of, but slightly removed from, the odontoblastic layer and, with time, was seen farther and farther from this region (FIGURE 19, ascending arrows). In animals carried to 10 days following injection, it was felt that the band of reactive mineral became progressively less distinct, and ultimately was replaced by a diffuse type of reaction.

More recent investigations by Kumamoto (1953a, b) utilizing both radioactive calcium and radioactive phosphorus in newborn and three-day-old rats, have shown that in both cases the radioautographs revealed the existence of similar accretion bands due to the apposition of layers of labelled material. Thus, using the more precise radioautographs obtained with Ca45, it was possible to demonstrate the initial accumulation of dentinal minerals at the junction of predentin and dentin (FIGURE 13). In serially sacrificed animals which were three days of age at the time of administration of the isotope, the zone of labelled mineral of the incisor dentin was seen to be progressively displaced away from predentin (FIGURES 13 to 15), never, however, reaching as far as the dentino-enamel junction. In point of fact, a constant distance was always maintained between the dentinal reaction band and the dentino-enamel junction, but the band became somewhat more diffuse at later intervals (up to eight days following injection, FIGURE 15).

Bélanger, Lotz, Visek, and Comar (1954) followed accretion bands labelled with Ca45 in the dentin of growing pigs for long intervals of time after administration of this isotope (FIGURES 16 and 17).



FIGURES 13 to 15. Radioautographs of the incisor tooth in rats injected with Ca^{45}-chloride at birth and sacrificed two hours (FIGURE 13), three days (FIGURE 14), and eight days (FIGURE 15) after injection. undecalcified sections, coated to locate a plastic strip, except FIGURE 15, which is unstained. from Y. Kumamoto, 1953) ×90.

At two hours (FIGURE 13), the band of reaction is strictly limited to the dentin (D) although bordering predentin (PD). A gradient of reaction spreads from the band throughout the dentin. The enamel reaction is barely visible.

At three days (FIGURE 14), the band of reaction is about halfway through the dentin. At eight days (FIGURE 15), the band is located farther from the pulp and appears to be more diffuse than at earlier time intervals.

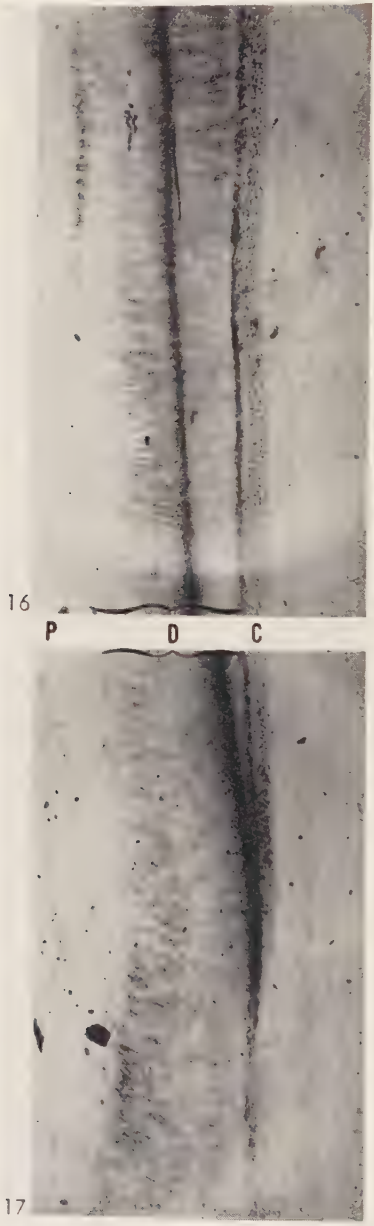
These and other unpublished data make it possible to conclude that minerals enter the dentin of young animals in several ways:

(1) The strong radioautographic band initially observed in dentin along the predentino-dentinal junction indicates the major area of mineral deposition. *At this junction, new layers of minerals are continuously added to dentin by a process of accretion* — a phenomenon which continues in adult animals, in which, however, the rate of mineral deposition is slower than in young animals (G. W. Wilkinson, 1949; L. F. Bélanger, 1952).

(2) The diffuse radioautographic reactions observed in dentin are due, in part, to the addition of some mineral throughout dentin, at least in very young animals. The concept of an *interstitial increase in the mineral content of dentin* is further demonstrated by the progressive increase in the hardness of dentin which is known to occur during the first few days after this layer appears in the tooth bud.

(3) Finally, in young rats, the main radioactive layer may show a decrease in sharpness with time (FIGURE 15), and pronounced changes in pattern have even been reported (L. F. Bélanger, 1952). Such variations in the arrangement of dentinal salts have been attributed to the *direct effect of mechanical factors* (pressure and tension) on the solubility of the crystals of dentin, since such factors would account for the dissolution and reprecipitation of minerals in limited areas (R. E. Glegg and C. P. Leblond, 1953).

Conclusion. Comparison of the data obtained from radioautographic study of elements of both matrical and mineral components of dentin points to the predentino-dentinal junction as a site of critical changes. In this area, two main phenomena occur: (1) a sulfated mucopolysaccharide, and probably another carbohydrate as well, are added onto the collagen base of predentin; (2) concomitantly, calcium and phosphate ions are deposited as dentinal crystals. The inference is that dentinal crystals are bound to the collagen base by car-



FIGURES 16 and 17. Radioautographs of the root of the incisor in a young pig injected with $Ca^{45}Cl_2$ and sacrificed *45 days* later (undecalcified section; inverted technique; basic fuchsin stain; from Bélanger, Lotz, Visek, and Comar, 1954). From left to right, pulp (P), dentin (D) and cementum (C) are seen. ×25
The oblique band of reaction separates dentin into two areas, that existing before administration of $Ca^{45}Cl_2$ (right-hand side) and that formed after injection of this element (left-hand side).

bohydrates, when a critical amount of suitable carbohydrates is reached as a result of the transformation of predentinal into dentinal matrix.

Enamel

In growing teeth, the area beween the layer of active ameloblasts and the fully calcified enamel shows successive zones, which correspond to progressive steps (or stages) in the mineralization of the matrix. These zones (FIGURE 1) are best demonstrated by Mallory or Masson trichrome and are as follows:

(1) *Pre-enamel* —the (blue staining) zone in direct contact with active ameloblasts or their Tomes fibers;

(2) *Young enamel* —the (red-staining) zone in which calcification begins;

(3) *Transitional enamel* —the (blue-staining) zone characterized by a gradient of calcification from the sparsely calcified young enamel to the fully calcified mature enamel (and by a concomitant decrease in the intensity of the blue stain);

(4) *Mature enamel* —the (unstained) zone which is fully calcified. This zone dissolves when the tooth is decalcified by standard procedures.

Formation of organic matrix. Two radioactive elements, S35 and C14, have been seen to enter the enamel matrix. Within a few hours after administration of *S35-sulfate* to rats and hamsters less than 12 days of age (L. F. Bélanger, 1954a), the pre-enamel exhibited an intense bandlike reaction (FIGURES 4 and 6, descending arrow) located in close contact with the apical region of the larger ameloblasts. No reaction was seen next to the smaller ameloblasts, some of which are young cells at the base of the root (FIGURE 4, lower left corner), while others are old cells at the apex of the cusp (FIGURE 1, above the descending arrow).

The reaction band present at two hours after S35 injection extended over a considerable length in teeth observed at early stages of their development, since it included apical and basal portions of the amelogenic organ. In contrast, the band was short in older teeth with well-advanced amelogenesis, since it was then limited to the more basal portion of the root. Finally, in teeth in which the formation of pre-enamel had ceased, for example, in the premolars of 12-day-old rats, no reaction band was seen in the vicinity of the ameloblasts at two hours after S35 injection.

In young rats sacrificed one day after S35 injection, the reaction band was still intense, but was near the junction of pre-enamel and young enamel. Two days after injection, the band of reaction was contained completely in young enamel, where it displayed the same length, but was broader, lighter, and more diffuse than at earlier time intervals (FIGURE 5, descending arrow). Apparently, the labelled material was dispersed over a broader area than it had occupied when deposited in pre-enamel. At this time (two days), the band of reaction was separated from the ameloblasts by a light, unreactive area corresponding to the most recently elaborated pre-enamel, in which, therefore, no radiosulfate had been deposited (this was to be expected since it was known that radiosulfate is rapidly eliminated from the circulation by fixation in tissues or by excretion).

At later intervals, the intensity of the reaction progressively decreased as the material composing the band became transitional enamel. With time, the reaction eventually vanished (FIGURE 7), suggesting that none of the sulfated material was retained in mature enamel.

Since pre-enamel contains no mineral elements, it was likely, from the outset, that radiosulfur was incorporated in the matrix. This inference was proved to be the case by showing that the reaction was not decreased after decalcification in acids (L. F. Bélanger, 1954a).

The nature of the sulfur-containing material of enamel is not known. Its insolubility in hyaluronidase (L. F. Bélanger, 1954a) would suggest that it is not chondroitin sulfate. Even so, it may, as in other locations, be a sulfated polysaccharide (L. F. Bélanger, 1954a).

The initial entry of small amounts of *C14-bicarbonate* into pre-enamel was observed by Greulich (1953b). Later, a rather wide and indistinct band of reaction was found in the areas of the matrix corresponding to young and transitional enamel. Again, the material disappeared during the last stages of calcification. The chemical nature of the C14-labelled material of enamel, protein, or carbohydrate, or both, is unknown.

While the amount of C14-labelled material appeared to be relatively less abundant than that labelled with S35, the behavior of the sulfur- and carbon-labelled materials during the various stages of enamel formation was similar; that is, initial deposition in pre-enamel, diffusion during transformation to young and transitional enamel and, finally, progressive disappearance as calcification became complete. The disappearance of labelled matrix reflects the loss of organic elements believed to take place in developing enamel as maturation proceeds.

Formation of enamel minerals. The deposition of mineral elements was examined with radiophosphorus and radiocalcium. Enamel formation was first investigated in the hamster by means of radiophosphorus (L. F. Bélanger and C. P. Leblond, 1950). Shortly after administration, the radioactive material was deposited in the enamel matrix at some distance from the ameloblasts. No reaction was observed in pre-enamel. The deposition was slight in the young enamel, but became progressively more intense throughout the transitional zone, reaching a maximum close to the fully mineralized enamel. Thus, there was a broad band of reaction, the edges of which were diffuse, particularly on the side facing the ameloblasts. With time, there was no significant change in the pattern.

In the rat, too, the deposition of radiophosphorus occurred as a gradient from the young enamel to the end of the transitional enamel, where deposition was maximal (L. F. Bélanger, 1952; see FIGURE 19), while no reaction was seen in fully mineralized enamel (FIGURE 19, M). The constantly growing incisor tooth in which the successive zones follow one another from the root outwards clearly showed the absence of reaction in pre-enamel, the reaction gradient extending throughout young and transitional enamel, and the rapid decrease of the reaction in the older tissue of the tooth apex, with its complete absence in mature enamel.

It is known that enamel is formed of crystals which increase in size during

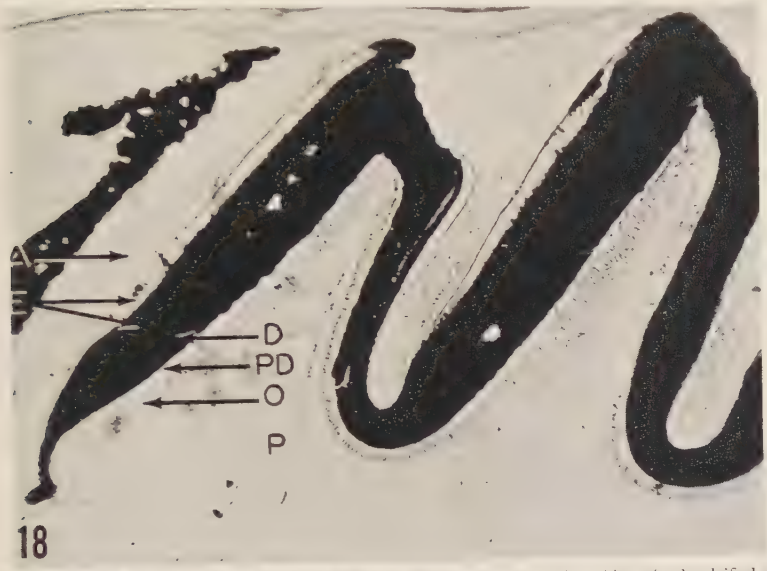


FIGURE 18. Von Kossa stain of the first cusp of the first molar of an eight-day old rat (undecalcified section). ×52.

The predentin (PD) does not stain. The dentin and enamel stain intensely. The unstained portion of the enamel corresponds to both pre-enamel and young enamel.

amelogenesis. The nuclei of these crystals appear at the young enamel stage and grow to full size during the transitional stage. The enlarging crystals offer a progressively increasing surface area for the deposition of more calcium and phosphate ions. This may explain the increase in the rate of mineralization as the crystal progresses through the transitional stage.

Conclusions. The main conclusions of this work have been deduced from observations made in the transitional zone. In this region, radioautographs produced by S35 and C14 show a decreasing gradient of intensity (parallel to the decrease in the amount of stainable matrix seen in histological sections). At the same time, the radioautographs of P32 and Ca45 show an increasing gradient of intensity. The contrast between the behavior of matrical and mineral elements may be due to the interaction of two factors: (1) mineralization may be associated with metabolic transformations leading to a loss of sulfur- and carbon-labelled compounds; (2) whatever matrix remains, it is dispersed by the accumulation of mineral elements, since the enamel crystals progressively grow until they make up approximately 95 per cent of the mature enamel.

Bone

According to the mode of development, it is possible to distinguish between endochondral and membranous bone formation. In the first case, bone arises from cartilage. Accordingly, the first part of this section will be used to present radioautographic data on the formation of *cartilage*; the second part will be devoted to the formation of bone *matrix*; and the third, to the deposition of bone *minerals*. In the fourth and last section, it will be recalled that the loca-

FIGURE 19. Radioautograph of the first cusp of the first molar in a rat injected with P32-phosphate at the age of four days and sacrificed *four days* later (undecalcified section; inverted technique; basic fuchsin stain). ×52

The dentinal reactions are narrow bands indicated by ascending arrows in the first and second cusps. The enamel reactions are located in the transitional enamel next to the dentino-enamel junction (descending arrows) and spread throughout transitional and young enamel as a decreasing gradient. However, in the second cusp—which is at a more advanced stage of development than the first cusp—the tip shows a portion of mature enamel (M) which has not picked up any radiophosphorus, as it is fully calcified.

tion of newly deposited matrix and minerals reveals some aspects of the *mechanics of bone growth.*

Cartilage formation. When *C14-bicarbonate* was administered to newborn rats, the cartilage of various areas such as trachea, articular cartilage, and epiphysis of long bones showed intense radioautographic reactions, which, however, exhibited pronounced differences in their pattern and intensity depending upon the time after injection (R. C. Greulich and C. P. Leblond, 1953).

The reactions were most pronounced in the hypertrophic cells of the epiphyseal cartilage and will be described in detail in this structure. One and four hours after the administration of C14-bicarbonate to newborn rats, radioautographic reactions were observed in cartilage lacunae and these were limited to the cytoplasm of the chondrocytes present (FIGURES 20 and 21). The cellular reactions of the epiphyseal cartilage showed a gradient of intensity. They were least in the zone of proliferating, palisaded cartilage cells farthest from the medullary cavity and increased as the medullary cavity was approached and the cells became hypertrophic. However, the cells nearest the medullary cavity, that is, those showing degeneration, exhibited no reaction, thus giving rise to an unreactive band in the region adjacent to the metaphyseal spicules (note the light band across the middle of FIGURE 20). The reaction of the hyaline matrix itself at these intervals was minimal throughout all cartilaginous structures. No significant reaction was observed in the perichondrium at these or any subsequent time intervals of examination.

FIGURE 20. Radioautograph of the epiphyseal portion of a long bone in a rat injected at birth with C14-bicarbonate and sacrificed one hour later. Undecalcified section, coated technique, unstained; retouched; from R. C. Greulich and C. P. Leblond, 1953). ×46

Cartilage is visible in the upper half of the figure. The dots of various sizes and shades are radioautographic reactions of single cells. While this type of reaction is visible throughout the epiphyseal cartilage, its intensity is maximal in the zone of endochondral ossification, except in its lowermost portion (degenerating cartilage) which gives little or no reaction. The uneven area at the top right is due to degenerating cartilage cells in a recently formed ossification center.

Some reaction may also be seen in subepiphyseal and periosteal bone spicules.

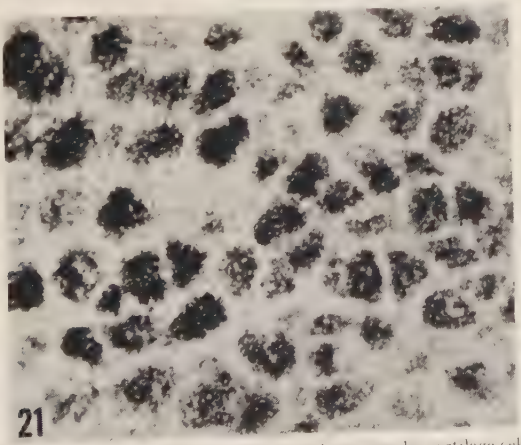


FIGURE 21. High power view of the radioautographic reactions given by cartilage cells in the same preparation as FIGURE 20. Approx. ×400

Note the intense radioautographic reaction arranged in definite spots, each one of which corresponds with the cytoplasm of a cartilage cell. The intercellular matrix shows little or no reaction.

FIGURE 22. Radioautograph similar to FIGURE 20 but in a rat injected at birth and sacrificed *24 hours* later. ×50

The cartilage shows a reaction in both cells and intercellular matrix. Note the intense reaction of the bone spicules.

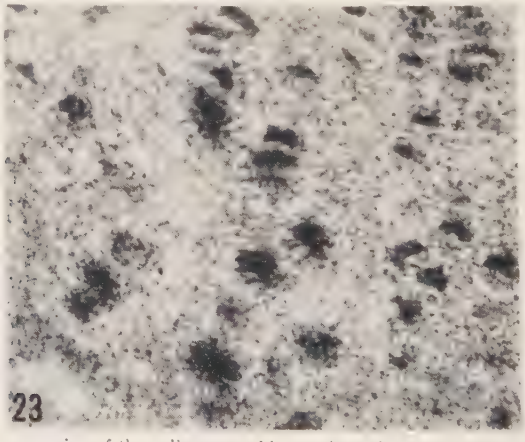


FIGURE 23. High power view of the radioautographic reactions of cartilage similar to FIGURE 21, but in a rat sacrificed *72 hours* after injection (technical conditions as in FIGURE 20, except for counterstaining with H. and E.). Approx. ×400

The cells are recognized in this photograph due to the staining of the cytoplasm with hematoxylin and eosin, but there is little radioautographic reaction in the cytoplasm. Most of the reactive granules are now present in the intercellular matrix.

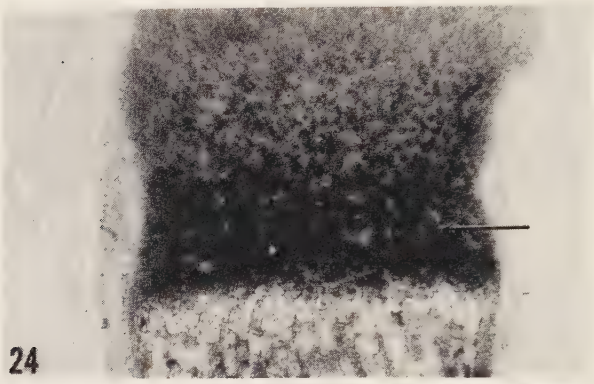
Scrutiny of similar areas of cartilage at intervals up to 72 hours after injection revealed a progressive decrease in the reactivity of the cartilage cells, whereas the hyaline matrix became progressively more and more reactive (FIGURES 22 and 23). The loss of C14 from the cells and its entry into the matrix were interpreted as indicating the elaboration by chondrocytes of a substance which, at later intervals, became incorporated into the matrix proper. These data, then, present direct evidence for the intercellular growth of the hyaline matrix of cartilage.

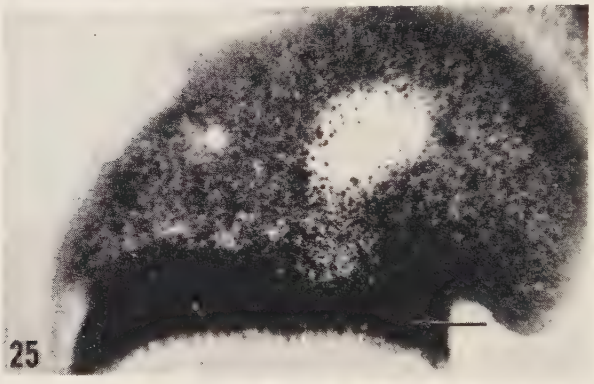
Since these data were obtained in sections of bone and cartilage which had been decalcified by routine histological methods and had, as well, been treated with saliva to remove glycogen, it appears that the carbon-14 incorporated into the cells and matrix of hypertrophic cartilage exists in the form of high molecular weight compounds of either protein or carbohydrate nature.

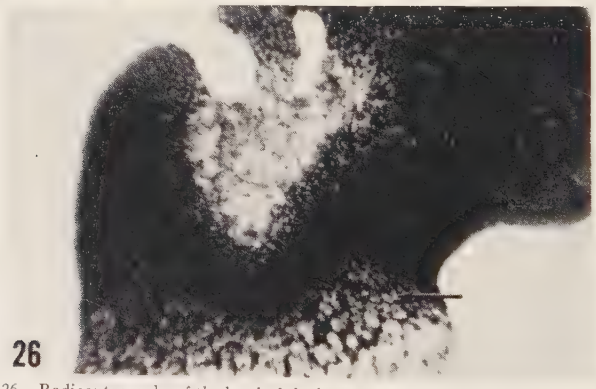
Dziewiatkowski (1951 and 1952), Boström and Odeblad (1953) and Bélanger (1953 and 1954b) have been the chief contributors to the study of growing cartilage with *S35-sulfate*. The first investigator demonstrated the uptake of radioactive sulfur in epiphyseal cartilage of young rats by the radioautographic technique. However, the contact method of radioautography which he used did not lend itself readily to precise localization of radioactivity. He noted that S35 was initially taken up throughout the entire epiphyseal cartilage, but that its concentration at early intervals was highest in the region of the epiphyseal-diaphyseal junction where the more mature cartilage cells were. Over the intervening time, between 24 hours and 12 days after injection, there was a tendency for this radioautographic distribution to become more uniform and there was, as well, a progressive loss of intensity.

Dziewiatkowski also reported that radioautographs of cartilage removed from similarly treated animals but fixed in a dilute solution of formalin saturated with barium hydroxide were almost entirely unreactive. This finding was explained as being the result of the formation of a soluble barium salt of chondroitin sulfate which was then released by the cartilage into the fixation medium. The inference was therefore drawn that most, if not all, of the radioactive sulfur taken up in epiphyseal cartilage was in the form of chondroitin sulfate.

Recently, Bélanger (1953 and 1954b), utilizing a more precise radioautographic method, demonstrated that the uptake of radioactive sulfate in growing epiphyseal cartilage occurs initially intracellularly (FIGURE 24) in a manner similar to that seen with carbon-14. At the earliest intervals examined; *i.e.*, one and two hours after injection of the tracer, the radioautographs revealed no significant uptake by the degenerating cells bordering the medullary cavity, while the next few rows of hypertrophic cells showed an intense uptake, which progressively decreased as the distance from the medullary cavity was increased. By 48 hours after injection, the reaction over the cells and over the matrix was approximately equal in intensity (FIGURE 25). At this interval, as well, the presence of radiosulfur became evident in degenerating cartilage matrix and even appeared over trabeculae of calcified cartilage beneath the epiphyseal plate. Six days after the introduction of radiosulfur, the radio-







FIGURES 24 to 26. Radioautographs of the head of the humerus in rats injected with S35-sulfate at the age of four days and sacrificed *one hour* (FIGURE 24), *two days* (FIGURE 25) and *six days* (FIGURE 26) later (inverted technique; basic fuchsin stain; from L. F. Bélanger, 1954b). ×32

At two hours (FIGURE 24), dots are seen which correspond to the radioautograph of a single cartilage cell. The darker dots (arrow) are those of the hypertrophic cells of the zone of endochondral ossification, which in its lowermost portion gives little or no reaction.

At two days (FIGURE 25), the cellular reaction is still visible, but in addition, a reaction of the intercellular matrix is indicated by the solid appearance of some reactive areas (arrow).

At six days (FIGURE 26), the reaction is exclusively present in the matrix; hence the cells appear as empty spaces (arrow) within it.

FIGURE 27 Radioautograph of the head of the humerus in a rat injected with S35-sulfate at the age of eight days and sacrificed one day later. The preparation was treated for three hours in a solution of *hyaluronidase* (inverted technique; maximal exposure; from L. F. Bélanger, 1954b). ×32

The radioactivity of the cartilage cells and matrix is almost completely removed by the treatment with hyaluronidase (the remaining areas may correspond to sites of insertion of muscles).

autographic reactions were present exclusively in the matrix. The cells were seen as light areas in the reactive matrix (FIGURE 26). At that time, some of the radioactive trabeculae of calcified cartilage were covered by layers of bone, thus forming the bony spicules associated with the epiphyseal plate.

The intense uptake of S35-sulfate by cartilage was recently confirmed (D. V. Davies and L. Young, 1954). These authors emphasized the fact that the S35 material present decreases in amount with time and attributed this phenomenon to a continual replacement of the S35-labelled tissue constituents.

Treatment of cartilage sections with hyaluronidase preparations (L. F. Bélanger, 1954b) prevented the S35-reactions of cells and matrix more or less completely (FIGURE 27). These findings were interpreted as indicating that the substance formed from radioactive sulfur was chondroitin sulfate, thus substantiating the conclusions of Dziewiatkowski.

When the isotopes which may be deposited as bone salts, that is, P32-phosphate and Ca45 ions, were administered, no significant uptake was observed in ordinary cartilage. However, an intense deposition was recorded in the zone of degenerating cartilage (C. P. Leblond, G. W. Wilkinson, L. F. Bélanger, and J. Robichon, 1950. See the dark band on the lower surface of the epiphyseal plate in FIGURE 39). It is of interest to note that the cells of this area did not elaborate the C14- and S35-labelled components of the matrix (as shown by the light band in FIGURES 20 and 24). Since these cells are in fact dying or dead, it looks as though cell death makes possible the calcification of cartilage matrix.

In conclusion, the following sequence of events may be suggested on the basis of the available data:

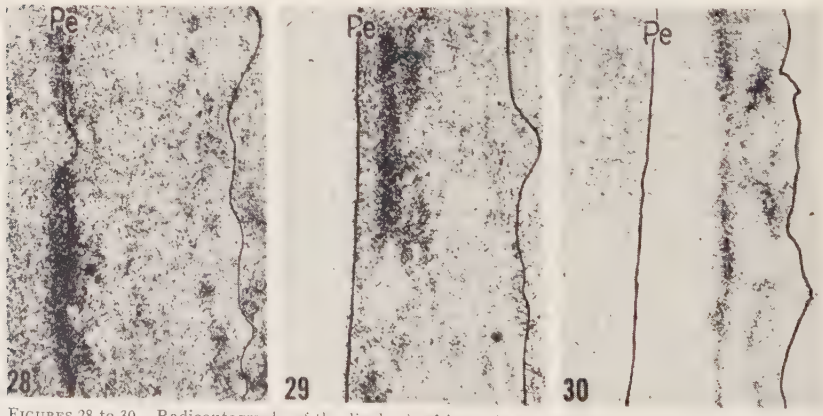
(1) Materials arising from C14-bicarbonate (collagen or other matrix material?) and S35-sulfate (chondroitin sulfuric acid) appear in chondrocytes and

later migrate to the matrix. Therefore, the chondrocytes elaborate these materials in their cytoplasm before they secrete them into the matrix.

(2) In epiphyseal cartilage, the rate of deposition of C14 and S35, that is, the rate of matrix formation by a given cell is related to the size of this cell. The addition of material by a cell to the matrix ceases when that cell degenerates, but the matrix then acquires the ability to calcify, and to serve later as framework for the deposition of layers of bone. This sequence is the process of endochondral bone formation.

Formation of bone matrix. The formation of the matrix was investigated radioautographically by the use of radioactive carbon and radioactive sulfur. Studies of *C14-bicarbonate* metabolism (Greulich, 1953a, b) have indicated that the matrix, but not the mineral elements of bone, were labelled in very young animals. This finding was demonstrated by the fact that the distribution or intensity of the radioautographic image was not modified by acid decalcification. The radioautographic observations of the matrix gave the same results whether the layers of bone were accumulated on cartilaginous spicules, as in endochondral bone formation, or appeared within connective tissue, as in membrane bone formation. In all cases, the reactions at four hours after injection lay in a position intermediate between the osteoblasts and the existing matrix. Thus, in the periosteal region in the middle of the shaft of long bones, an irregular reaction band was found at the junction between the osteogenic cells and the matrix (FIGURE 28). This intermediate position of the reaction band suggests the existence between the osteoblasts and bone matrix of a thin layer of matrix precursor (preossein, osteoid) which would function in the same manner as predentin does as precursor of dentin. The existence of this preossein remains hypothetical here, since it could not be clearly seen in the material used.

At later intervals after the administration of isotopic carbon, the labelled



FIGURES 28 to 30. Radioautographs of the diaphysis of femur in rats injected at birth with C14-bicarbonate and sacrificed *four hours* (FIGURE 28), *one day* (FIGURE 29) and *three days* (FIGURE 30) later (coated technique; unstained). Approx. ×200

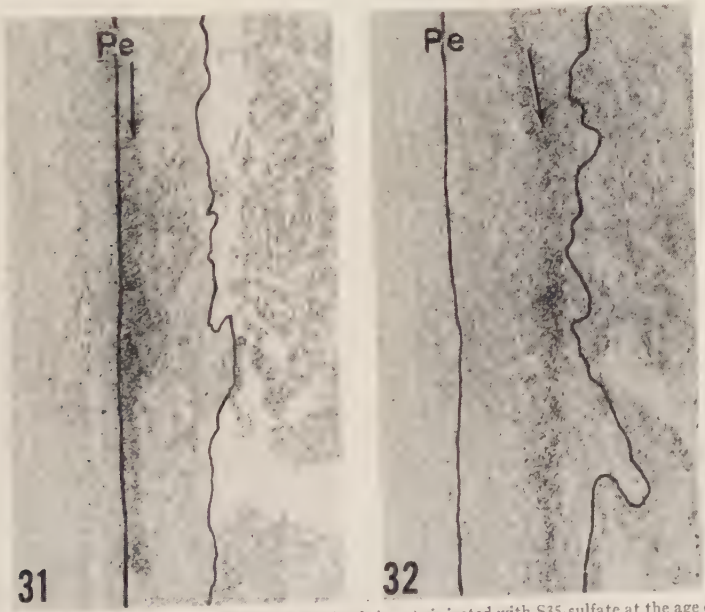
A comparison of the figures shows that at four hours the periosteal region (Pe) is reactive (FIGURE 28), at one day the reaction is present at some distance from the periosteum (FIGURE 29), while at three days the reaction is present at a much greater distance from the periosteum (FIGURE 30) than at the earlier time intervals.

matrix was seen to be caught up in the substance of the bone as an irregular band of reaction lying at a progressively increasing distance from the osteogenic cells (FIGURES 29 and 30).

Finally, radioautographic reactions of membrane bone were seen in areas of mesenchymal tissue where characteristic osteoblasts had arranged themselves in the typical pattern of osteogenic tissue, but no morphologically distinguishable bone had as yet been laid down. It was felt that, in this case, the formation of osseous organic matrix had been visualized just before the process of mineralization had actually begun.

In regard to the localization of *S35-sulfate* in the matrices of periosteal and membrane bone, the first indication of uptake of this isotope in bone was again offered in the radioautographic work of Dziewiatkowski (1951). However, these studies did not establish whether the labelled component was part of the minerals or of the organic matrix.

Later studies made on young animals sacrificed at intervals up to four days after injection established that S35-sulfate was incorporated in the matrix (L. F. Bélanger, 1953, 1954b). At very early intervals after radiosulfur administration, undecalcified bones displayed a generalized diffuse type of radioautographic reaction throughout the thickness of the periosteal bone. This diffuse pattern was eliminated following acid demineralization, but a band of radioactive material persisted immediately beneath the periosteum (FIGURE 31). This band was taken to represent a zone of labelled organic matrix, while the



FIGURES 31 and 32. Radioautographs of the diaphysis in rats injected with S35-sulfate at the age of four days and sacrificed two hours (FIGURE 31) and four days (FIGURE 32) after inverted technique; basic fuchsin stain; L. F. Bélanger, 1954b). ×110

The reactions (arrows) of the bone matrix appear at first in the area of the periosteum (Pe) and are later found at some distance away (FIGURE 32). The distance is an index of the amount of bone elaborated during the interval between injection and sacrifice.

diffuse reaction seen in bones prior to decalcification was interpreted as representing a generalized incorporation of inorganic sulfur into bone minerals by exchange or adsorption at the surface of the bone crystals.

In animals sacrificed four days after injection, autographs of undecalcified sections showed that this growth band persisted and was now well within the shaft (FIGURE 32), while the diffuse mineral reaction had vanished (presumably by reverse exchange as in the case of dentin).

The sulfur reactivity of the matrix, like bone metachromasia, could be removed by treatment with hyaluronidase. It is, therefore, likely that, in bone, as in dentin and cartilage, the S35-labelled matrix component is chondroitin sulfate (L. F. Bélanger, 1954b).

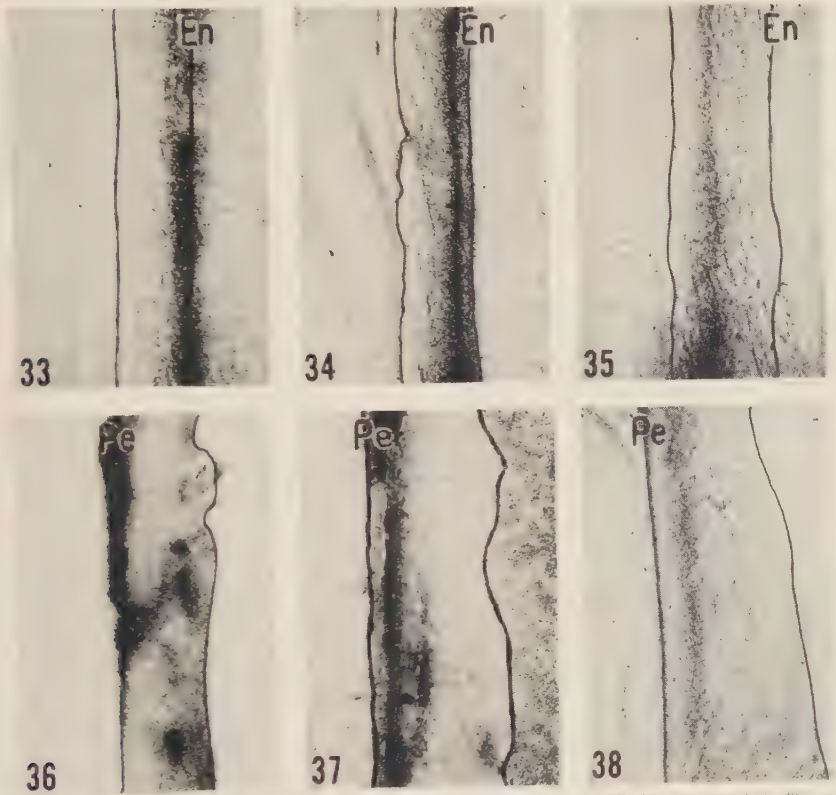
In conclusion, the data so far obtained indicate the incorporation into the matrix of components containing carbon (collagen?) and sulfur (chondroitin sulfate). Although the spatial relationships are not as clearly seen as in dentin, it is believed that, here too, the C14 is first present in a precursor of the matrix (preossein) which very soon incorporates chondroitin sulfate and is thus transformed into true matrix capable of fixing mineral salts.

Formation of bone minerals. It has been pointed out above that "bone salts," that is, calcium phosphate crystals may be deposited directly in degenerating cartilage. Furthermore, this cartilage serves as framework for the deposition of bone layers (endochondral bone formation). In other types of bone formation, layers of bone appear directly in connective tissue (membrane bone formation, periosteal bone formation). In all these cases, observations carried out in young animals reveal that mineralization proceeds along two pathways: (1) an accretion of new layers of minerals, appearing in radioautographs as reactive growth bands; (2) a widespread entry of material through the thickness of bone, giving rise to diffuse reactions.

The extent of the two types of reactions varies according to the age of the animal under investigation (C. P. Leblond, G. W. Wilkinson, L. F. Bélanger, and J. Robichon, 1950). In newborn rats and cats, the bone radioautographs initially showed both diffuse reactions and bandlike localizations. What little banding did occur was observed in the periosteal region and on the surface of the subepiphyseal spicules. Some of the diffuse reactivity persisted for a time. This persistence was attributed to the interstitial deposition of bone crystal material. This fact clearly demonstrated in newborn animals became less and less pronounced as the animals grew older.

In growing rats (50 gm.), there was a less intense diffuse autographic reaction, but the band reactions were now quite definite (FIGURES 36–38). Thus, the minerals were deposited mainly as layers (generally located in close contact with osteoblasts). At later time intervals, the reactive bands were found farther and farther from the osteoblasts as new minerals (which by this time were no longer significantly labelled) were added by the activity of these cells. As a result, the band moved away from the osteoblasts and eventually could even reach the opposite surface of the bone where erosion took place.

It may be concluded that, in bone as in dentin, both the processes of accretion and interstitial deposition of minerals took place. Interstitial deposition was



FIGURES 33 to 38 Radioautographs of the funnel (FIGURES 33 to 35) and cylinder (FIGURES 36 to 38) region of the tibia diaphysis in rats sacrificed four minutes after intravenous injection of P32 phosphate. ×29.
FIGURES 33 and 37 and 8 days. FIGURES 35 and 38 after intraperitoneal injection of P32 phosphate. ×29.
In the *funnel region* the reaction band appears on the endosteal surface (FIGURE 33) and is displaced towards the periosteal (Pe) surface (FIGURES 34 and 35).
In the *cylinder* region the reaction band appears on the periosteal (Pe) surface (FIGURE 36) and is displaced towards the endosteal surface (FIGURES 37 and 38).

significant in very young animals, but later accretion became the main factor of growth.

Mechanics of bone formation. Accretion bands may be observed in radioautographs of matrix (FIGURES 28 to 30, and 31 and 32) and minerals (FIGURES 36 to 38). In growing rats (50 gm.), these accretion bands were found essentially in three locations: (1) at the external surface of the mid-diaphysis, that is, where periosteal bone formation takes place (FIGURE 36); (2) at the endosteal surface lining the funnel-shaped subepiphyseal portion of the shaft (FIGURE 33); (3) at the surface of the endochondral spicules located within the epiphysis and on either side of the epiphyseal plate. This was first shown with the help of P32-phosphate (C. P. Leblond, G. W. Wilkinson, L. F. Bélanger and J. Robichon, 1952) and recently confirmed by following C14 in matrix (R. C. Greulich, 1954).

The material added at the surface of the mid-diaphysis, or *cylinder*, con-

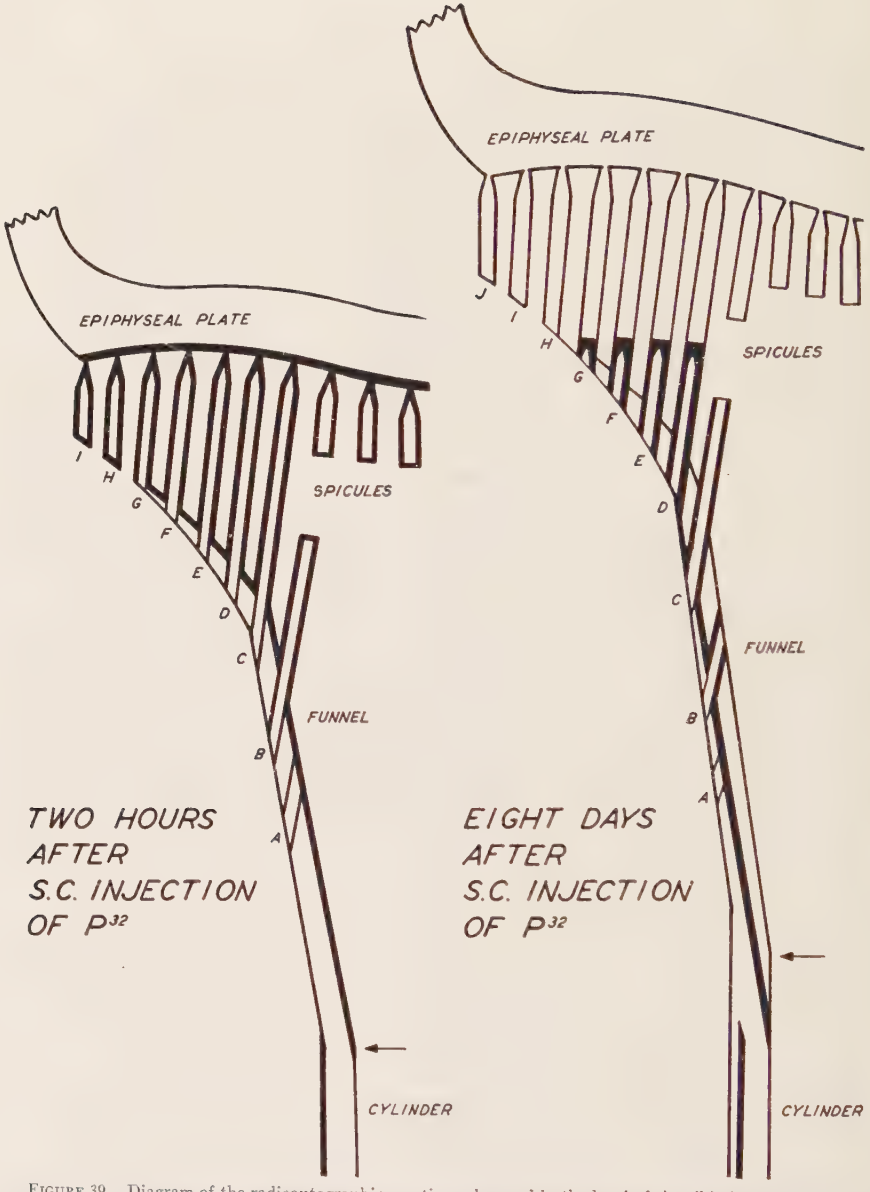


FIGURE 39. Diagram of the radioautographic reactions observed in the head of the tibia in 50 gram rats injected with P32-phosphate and sacrificed two hours (left) or eight days (right) later (coated technique; from C. P. Leblond, G. W. Wilkinson, L. F. Bélanger, and J. Robichon, 1950).

The heavy lines indicate the areas of deposition of radiophosphorus. These are drawn at the same level in both diagrams.

The reactive line found in the calcified cartilage on the lower surface of the epiphyseal plate soon after injection (left) persists as a broken line across some of subepiphyseal spicules (right). The rest of the line was eroded. The reactive coating found on the spicules soon after injection persists on the lower parts of some of them (right); by tracing the individual spicules from one diagram to the other their contribution to the wider end of the funnel becomes apparent. The reactive line found on the endosteum of the funnel soon after injection (left) becomes deeply embedded in the bone along most of the length of the funnel and is even reaching the outer surface at the wider end (right). The reactive line found on the periosteal surface of the cylinder soon after injection (lower left) becomes embeded in the bone (lower right).

This pattern of deposition of radioactive phosphorus was recently found to hold for the deposition of radioactive carbon in the matrix.

sisted of periosteal bone. Radioautographs of serially-sacrificed preparations showed an apparent movement of the newly formed layer of bone toward the endosteal surface (FIGURES 36 to 38). This movement resulted from the addition of new layers of material at the periosteal surface and the simultaneous resorption of old layers from the endosteal surface. This observation was in full agreement with the classical theories on the formation of periosteal bone in this region of the diaphysis.

The walls of the subepiphyseal region, or *funnel*, exhibited an addition of bone on the endosteal surface (the so-called "funnel effect"). The successive addition of material on the inner surface of the funnel resulted in a burial of the radioactive band within bone tissue (FIGURES 33 to 35). In this region, bone was resorbed by osteoclasts from the external surface; indeed, the distance between the radioactive band and this surface became smaller with time (FIGURE 35). The successive addition of layers on the inner surface of the funnel results in a type of growth which may be represented by the gradual rise of an inverted truncated cone, attached by its narrow end to a cylinder. The detail of this process as seen after P32 injection is illustrated in FIGURE 39.

Conclusions

When radioautography is carried out by one of the "integrated techniques," such as the fluid-coating or inverting technique, fine details of matrix formation and mineral deposition may be seen in developing hard tissues.

In the case of *enamel*, there is a clear-cut discrepancy between the appearance of matrix and minerals, since the deposition of matrical components, as visualized with S35-sulfate and C14-bicarbonate, occurs long before minerals (P32-phosphate, Ca45 ions) are deposited. In fact, the matrical labels disappear when and where the mineral labels are accumulated.

In the case of *cartilage*, matrix formation includes an incorporation of carbonate and sulfate, but the substances containing these ions are here synthesized within the cytoplasm of cartilage cells and later deposited in the matrix. Calcification occurs only in degenerating cartilage, in which matrix formation has ceased.

In the case of *dentin* and presumably also *bone* the matrix is elaborated in two steps: at first, predentin (or preossein) is formed with incorporation of C14-bicarbonate. Addition of S35-sulfate as chondroitin sulfate contributes to the transformation into true dentin (or ossein) matrix. Such matrix is capable of taking up phosphate and calcium salts as dentinal (or bony) crystals.

Except for an interstitial deposition of minerals in the dentin and bone of very young animals, the addition of minerals, as well as the formation of matrix, takes place by apposition of successive layers, that is, by accretion. Tracing the fate of these layers (seen as bands in radioautographs) clarifies the mechanics of the growth of dentin and bone.

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THE NATURE OF THE BONE SALT

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Constituents of bone and phosphorites other than those of hydroxyl apatite, $Ca_{10}(PO_4)_6(OH,F)_2$, are now known to be present on terminating surfaces of crystals. The most significant of these are CO_3^{--} or HCO_3^-, H^+, excess $(OH)^-$ and F^-, and H_2O with minor Na^+, Mg^{++} or $Mg(OH)^+$, citrate, *etc.* The composition within the unit of structure in bone is essentially fixed without substitution of Ca^{++} by Na^+, as might be expected from equivalence of ionic radii, or of Ca^{++} by Mg^{++}.

The surface constituents can be of the order of 10 per cent of the total composition of bone, which is many orders of magnitude greater than that for most other materials. Reasons for this condition must be largely dependent upon the atomic arrangement at the surface of the apatite crystals. These arrangements are examined in detail and are illustrated. The peculiar relations to carbonate and to hydrogen ion, which are the most determinative ones for bone, are evident. Warrington's octacalcium phosphate, which is the apatite with the lowest $Ca:PO_4$ ratio and a limiting model for the bone salt, has surface hydrogen ions forming acid phosphate groups. Surface compositions are discussed in detail insofar as they are structure-controlled.

Influence of these surface factors and of the internal atomic arrangement on crystal growth and shape are discussed. Reasons for the hexagonal axis lying in the extended prism face are evident (Abstract).

SOME ACHIEVEMENTS AND PROBLEMS IN STUDYING THE SOLUBILITY OF THE MINERAL OF THE HARD TISSUES*

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"A knowledge of the factors affecting the precipitation and solution of calcium carbonate† and calcium phosphate underlies the solutions of such physiological problems as bone and tooth formation and such pathological problems as rickets and arteriosclerosis."[1] This statement gives a clear directive to turn toward the relatively simple physicochemical problems in the calcium-phosphate-water system to gain some understanding of the phenomena of precipitation and solubility. In the present brief review, it is our intention not to consider the multiple complications unavoidably present in the biological system, and to forget, for the moment, that vitamin D, various hormones, conditions of oxygenation, and degree of CO_2 saturation, for example, all may play important roles *in vivo*. Ultimately, the interaction of these factors must be all woven into the story of the growth, the health, and the diseases of bones and teeth. For a start, however, even the comparatively simple process of the formation of hydroxylapatite *in vitro* must be described as a mystery. The solution process also is so complicated that only recently reproducible values have been obtained.

One conclusion can be drawn: *there is no solubility product for hydroxylapatite*. For macromolecules like solid sodium chloride, the simple relationship of the product of the ion activities is a constant. The crystal of bone mineral, hydroxylapatite, is not in this sense a macromolecule. It is really not a simple chemical compound. Hydroxylapatite is a crystal lattice in which isomorphic substitutions produce a variable composition from part to part of the crystal. *The solubility of such a crystal cannot be expressed as a solubility product*. All of the attempts to calculate a solubility product for a basic calcium phosphate have resulted in numbers referred to as "constants" that varied not by tenfold or one hundredfold, but by one millionfold or more. From our present understanding of the nature of the bone mineral, this variability is to be expected. No theoretical grounds for expressing the solubility by the classical calculations of the product of ion activities can be given.

The inability to describe the solubility in simple terms does not mean that there is no relation between the concentration of the constituent ions; *e.g.*, calcium and phosphate ions, in the extracellular fluid and the mineral phase. It is unimaginable that any but an equilibrium condition exists. There must be a moment-to-moment interchange of ions from the crystal surfaces into solution and from the extracellular fluid onto the crystal surfaces.

In vivo formation of the bone mineral, however, may not be quantitatively related to the precipitation of hydroxylapatite from solution.[2] Neuman and Neuman have recently suggested[3] that bone crystals begin as a catalyzed

* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

† Nowadays, it is no longer believed that calcium carbonate exists in bones and teeth of most species as a separate solid phase.

crystallization on an organic template that provides the exact spatial arrangement of the apatite lattice. This concept assumes that the extracellular fluid from which the bone forms has a high degree of constancy of composition and that the bone deposited from such a fluid has, therefore, a nearly constant composition. This concept represents a departure from the classical picture advanced by Robison[2] that the solubility of tricalcium phosphate was the regulating mechanism for calcification. It appears more likely that cellular mechanisms are responsible for final control of serum and extracellular fluid composition. Bone mineral formed from this solution has a very important role in *stabilizing* the concentrations of the ions at the levels fixed originally by the cells. The solubility of hydroxylapatite, therefore, should be thought of as a regulating mechanism for maintaining the constancy of the fluid composition. This mechanism is an important factor in homeostasis. Even with such a simple hypothesis, it is still impossible to describe how bone mineral is related to the ionic concentrations. The dictum of Hastings[1] is still valid: "apparently calcification does not bear a simple relationship to the calcium of the blood."

The Howland-Kramer calcium phosphate product. In 1923 Howland and Kramer reported in the *Monatschrift fur Kinderheilkunde*[4] values in milligrams per cent for calcium and inorganic phosphate found in the blood serum of children and of rats suffering from rickets or under treatment and healing. They found that "when the product of the concentrations of these two substances was less than 30, rickets was regularly present. When the product was more than 40, rickets was absent. Between 30 and 40 there was a beginning of repair of the bones or evidences of mild rickets." The thermodynamic significance of this product is not clear. The total calcium concentration is probably almost twice the calcium ion concentration, and most of the inorganic phosphate concentration at the pH of the blood is secondary phosphate. The product resembles the solubility product for secondary calcium phosphate but cannot be rigorously defined. The Howland-Kramer product is still in use, mostly because it is the only workable simple number that permits an inference to be drawn about the clinical status of calcification. On an empirical basis its value has been repeatedly demonstrated.

The success of the Howland-Kramer product in diagnosing rickets and in following the clinical status under therapy has been an important reason for the diligent but unsuccessful search for $CaHPO_4$ in bone. Every recorded attempt to show that $CaHPO_4$ is present has ended in failure. The most powerful modern tools such as electron spectroscopy, X-ray diffraction and optical analyses have never given evidence of even trace amounts of the secondary phosphate. This compound precipitates from aqueous solutions in well-characterized crystals of $CaHPO_4 \cdot 2H_2O$ under easily available conditions.[5]

When the pH of a solution containing sufficient amounts of calcium and phosphate is raised, these crystals form the first precipitate. If, then, there is an additional increase in pH to values like those of blood or extracellular fluid, the secondary calcium phosphate crystals slowly change over to the hydroxylapatite lattice, the mineral of bones and teeth. Although an exhaustive scrutiny has never revealed $CaHPO_4$ in bone, it appears that the solubility

product of this material is a numerical ceiling above which calcium phosphate ion activity products in the body never go.[6]

To summarize the evidence for and against the participation of $CaHPO_4$ in calcification, the following tabulation has been made.[7]

Con	Pro
$CaHPO_4$ crystallizes only from acid solutions.	$CaHPO_4$ is unstable at pH 7.3 and changes to a basic calcium phosphate.
Early calcification has a high, not a low Ca/P.	No calcification occurs when the ion product is much less than the $K_{s.p.}$ $CaHPO_4$.
No evidence of a $CaHPO_4$ X ray diffraction pattern *in vivo*.	Mixed precipitates of $CaHPO_4$ and hydroxyl apatite can be formed *in vitro* in acid solutions.
Pyrophosphate is not present in bone ash.	Rat bone formed on a low calcium-high phosphate diet empirically contained what was described as a $CaHPO_4$ apatite.

From the laboratory of experimental biology comes still another index. McLean's "biological solubility," a criterion of calcification, is the minimum ion product constant at which calcification occurs *in vitro* in slices of rachitic cartilage. In a graph of phosphate concentration against pH at constant calcium concentration, the curve for $K_{s.p.}$ $CaHPO_4$ was entered. The biological solubility fell on the alkaline side along the $CaHPO_4$ curve; the significance of this behavior is not understood.

The Howland-Kramer product is the only valid index of calcification *in vivo*. It may be related to and certainly superficially resembles a solubility product. The product, however, appears to describe an upper limit never achieved in blood plasma or extracellular fluid. Since it is probable that $CaHPO_4$ never forms at any time, there is no reason why the $K_{s.p.}$ of secondary phosphate should rigorously describe the relation between extracellular fluid and bone mineral. The anomaly lies in the success of the Howland-Kramer product as an index of the solubility or, at any rate, as an index of the active process of calcification in which the solid deposited is a more basic calcium phosphate, hydroxylapatite. Neuman has pointed out that the Howland-Kramer product indicates that the calcification process *in vivo* must be related to calcium and phosphate ion concentrations directly; *i.e.*, to the first power, despite the fact that the composition of the product formed does not contain calcium and phosphate in a 1:1 but in a 10:6 ratio. This is a case where a clinician's rule of thumb has withstood examination by a number of carefully planned and executed physicochemical and biological studies and still remains a mystery.

The nature of the mineral of bones in teeth. Before proceeding to discuss the solubility characteristics of hydroxylapatite, some of the characteristics that may influence or govern the solid-solution system can be indicated. The space lattice of hydroxylapatite has been computed[8] so that the location of each calcium, phosphate, and hydroxyl group is known with some precision. The approximate empirical formula is $Ca_{10}(PO_4)_6(OH)_2$. The smallest repeating

structure, therefore, must contain at least 18 atoms or ions. The unit cell is a complicated arrangement of intricate symmetry. The most careful measurements reveal small but significant variations in the dimensions of the unit cell from sample to sample. That these variations have a structural basis is made probable by the variations from exact composition found on chemical analysis. Hydroxylapatite should be thought of as a crystal of variable composition, with an indefinite series of nearly perfect isomorphous substitutions.

The calcium, phosphorus, hydroxyl, and hydronium ions must be assumed to be present in variable proportions in synthetic hydroxylapatite depending on (a) the composition of the liquid phase, (b) the time of equilibration, (c) the relative weights of solid phase to solution, and (d) whether the solution was originally CO_2-free or CO_2-controlled or -uncontrolled. In bones and teeth, in addition, the crystals have significant amounts of CO_2, magnesium, sodium, citrate, and organic constituents of unknown nature. To describe the solubility of such a complex and variable structure in simple terms is inherently difficult and, at present, impossible; the theoretical grounds have not yet been supplied. Investigators, therefore, have been satisfied to demonstrate that in broad outline the solubility of bone is like that of synthetic hydroxylapatite. Most studies have selected solution concentrations of calcium and phosphate approximating plasma concentrations and the pH range near that most interesting biologically (at least to human biologists); *viz.*, around 7.3. The fact that all samples of bone and tooth mineral ever examined by X-ray diffraction methods have shown a relative constancy of pattern by no means indicates an equal constancy of chemical composition. Precise statements, therefore, of the solubility relation *in vivo* may be impossible, although some of the important solubility characteristics are known. While it is literally true, as Neuman and Neuman pointed out in 1953,[3] that "bone does not conform to any known solubility relationship," the basic assumption is always made that there must be an effective dynamic equilibrium between the mineral of the hard tissues and the extracellular fluids that bathe them. The problem is to describe this equilibrium.

Types of solubility studies of the hard tissues. Solubility studies of enamel, dentin, cementum, and bone have brought out the essential similarity to the solubility of synthetic hydroxylapatite. Only a limited number of studies have been directed at the primary question of finding a number that will express the solubility of the hard tissue mineral. Several studies have been made of the solubility in acetate buffers. Solution rates have been recorded, but a thorough understanding of the solution composition, the nature and the concentration of each ionic constituent has not been fixed or described.[9] It is important to recognize the role various chelating agents in the tissue fluids may play. Extensions of the solubility studies have focused on alterations produced by treatment of the hard tissue (for example, enamel), with a variety of ions of which fluoride and tin might be cited as examples. A third kind of study has been conducted in which partial solutions have been prepared by leaching the hard tissues with repeated acid treatment.[10] In general, these studies of fractional solubility reveal complicated processes of solution, accompanied by hy-

drolysis and perhaps by reprecipitation. Specifically, the first acid treatment gives a solution high in calcium relative to phosphorus, high in carbonate, high in magnesium, and high in citrate. Similar trends have been found when synthetic mixtures of calcium carbonate and calcium phosphate were leached by the same acid procedure; whereas, the minerals, dahllite and staffelite,[11] described as carbonate apatites have considerably different courses. The solubility studies of the hard tissues have emphasized the complicated nature of the naturally occurring minerals and have underlined the need for a simpler approach. A number of investigators have taken up the study of synthetic hydroxylapatite solids in aqueous solutions containing calcium and phosphate ions.

Solubility studies of synthetic hydroxylapatite. The low solubility of the bone mineral confers upon this substance the ability to control in the body fluids the concentrations of some of the most important elements and groups involved in metabolic processes. For the solubility of hydroxylapatite, often sold commercially under the label, $Ca_5(PO_4)_3$, the *Chemical Rubber Handbook* gives a value of 2 mgm. per 100 ml. of water. More careful reading will show the inadequacy of such a number; for instance, the amount of dissolved material is notably dependent on the pH.

Several interesting facts about the solubility of hydroxylapatite can be drawn from a study of the changes that occur when phosphoric acid is titrated with calcium hydroxide.

Precipitation of $CaHPO_4 \cdot 2H_2O$. The pH, at first, rises slowly, and then very rapidly as the 4.5 end point is approached and exceeded. When the pH reaches 6.1, if the solution is stirred without further addition of calcium, tiny crystals having a characteristic tabular form accumulate slowly on the bottom of the beaker. These are $CaHPO_4 \cdot 2H_2O$ crystals. As the amount of precipitated secondary phosphate increases, there is a steady drop in pH.

Precipitation of hydroxylapatite. If more calcium hydroxide has been added before the crystals appear, so that the pH reaches 6.2, a different sequence of precipitation follows: the solution immediately becomes opalescent; the cloudiness soon changes to a milky appearance; and, in a few minutes, a flocculent precipitate seems to fill the entire volume. In this same brief time, the pH falls rapidly to about the same level attained after several hours by the solution in contact with the secondary phosphate crystals.[7] Both $CaHPO_4$ and hydroxylapatite precipitate from solutions in which the $HPO_4^{=}$ ion is present. In a titration process as described, $CaHPO_4$ and hydroxylapatite precipitate from solutions in which ion concentrations may be nearly identical.

The opalescence just described when examined by the electron microscope[12] shows, at first, amorphous fuzzy particles that give no evidence of any atomic orientation when tested by the electron diffraction method. Under certain conditions, these particles change their appearance in a few minutes and show sharp corners characteristic of the hydroxylapatite crystal habit in bone crystals but dissimilar to the geologic mineral apatite forms. The size and shape of these crystals depend, among other factors, on the relative amounts of calcium and phosphate ions in the solution. From a solution containing excess phos-

phate, the crystals tend to be long and thin whereas, from a solution containing excess calcium, the crystals appear to be nearly equilateral tablets. Freshly precipitated "hydroxylapatite" floc can be obtained in a highly hydrated form containing, for example, as much as 2000 per cent water.

In 1908 Bassett[13] analyzed solutions above precipitates of primary calcium phosphate and found the solubility curve relating Ca to P concentrations. In these systems, 100 gm. of solution contains 1 to over 3 gm. of calcium and 10 to 20 gm. of phosphorus. In slightly less acid solutions, he found that the solid phase was $CaHPO_4$ hydrate; 100 grams of these solutions contained 1 to 3 gm. of calcium and 2 to 10 gm. of phosphate. Decreasing the acidity still more, Bassett found a new solid phase that he described as $Ca_3P_2O_8$ hydrate. He drew, according to requirements by theory, a new solubility curve to fit these systems although, in his actual data, the points fell on an extension of the curve for $CaHPO_4$. In 100 gm. of solutions in contact with the basic phosphate, Bassett reported 0.01 to 0.04 gm. of calcium and 0.01 to 0.06 gm. of phosphorus. Hodge and Bale, 1938,[14] found that the concentrations of calcium and phosphorus in solutions in contact with hydroxylapatite fitted the $CaHPO_4$ curve of Bassett's even in highly alkaline solutions that contained in 100 cc. as little as 100 μg. of calcium and 10 μg. of phosphorus. The seeming ability of the secondary calcium phosphate curve for Ca versus P to describe the solid-to-solution relation for hydroxylapatite systems has not been satisfactorily explained. Even though the theoretical basis has not been supplied, the fact that there is a linear Ca to P relation for solutions in contact with hydroxylapatite extending from systems with 20 mg. of calcium to those with 100 μg. of calcium per 100 cc. and with 60 mg. of phosphorus to 10 μg. of phosphrous per 100 cc. gives evidence of a uniformity in behavior that is gratifying to find.

Calcium to hydrogen ratio. Shear and Kramer, in 1928,[6] observed that, in the solutions shaken with precipitated calcium phosphates, the calcium to hydrogen ratio was a constant. This is another indication of a uniformity in behavior of unknown origin. "The fact that the same calcium to phosphorus ratio and the same calcium and hydrogen ratio applies to solutions above secondary calcium phosphate crystals and to solutions above the flocculent precipitates of hydroxylapatite (or above powdered bone) may contain a clue as to the mechanism of calcification."[7]

Calcium to phosphorus ratio. The linear relation of calcium to phosphorus in solutions in contact with synthetic hydroxylapatite has been presented. The same relation holds reasonably well for systems in which the solid phase is powdered bone, although it was frequently found that, for a given phosphorus concentration, the solutions would contain more calcium than when synthetic apatites were studied.

When the original data of Howland and Kramer were recalculated and plotted in the same manner, their data also tended to show higher calcium concentrations for a given phosphorus concentration than the synthetic apatite solubility curve required. However, an interesting trend could be found when a boundary was drawn between "points for rachitic infants or rats and the

points for healing rickets or no rickets,"[7] the boundary line appeared to be at right angles to the trend line for the Ca to P relation of the synthetic apatites. Along this boundary line, the calcium phosphorus product was a constant numerically approximately equal to the $K_{s.p.}$ $CaHPO_4$.

Log calcium versus log phosphorus minus pH. By combining the two relations $\frac{Ca}{P} = k_1$ and $\frac{Ca}{H} = k_2$, a single equation can be obtained as follows: $2 \log Ca = \log P - pH + k_3$.[22] The calcium, phosphate, and pH data of a number of investigators have been computed in this fashion and a single line over a considerable range of pH gives a reasonable approximation of the trend. This line fits at least approximately when calcium concentrations varied from 10^{-1} to 10^{-5} M and over ranges of values of log P − pH of 11 log units.

Suspended solids. Investigators who have conducted solubility measurements on powdered bone have frequently been impressed with the marked and apparently uncontrollable variation in the concentrations of ions in systems stirred or shaken for days or weeks in an attempt to reach an equilibrium state.[15-21] From time to time, investigators plagued by the distressing variability in the concentrations of calcium and phosphate in solutions supposedly equilibrated with bone or with synthetic precipitates would be forced to conclude that there must be colloidal calcium phosphate in the solutions. The suggestions of the nature of this suspended material ranged from soluble, slightly ionized calcium phosphate complexes to suspended solids. Levinskas in 1953[23] satisfactorily demonstrated that there were in reality suspended solids that could be removed by ultracentrifugation or filtration through filters with very fine pores. He showed that filtrates containing no suspended solids gave reproducible calcium and phosphate concentrations. The dispersed material was characterized by a reasonably constant composition, not that of $CaHPO_4$, but apparently nearly identical with that of hydroxylapatite.

Levinskas and Neuman,[25] with the aid of this powerful technique, found that the solutions attained an apparent equilibrium with synthetic hydroxylapatite when shaken for as short a time as one day.

One of the anomalies that has been repeatedly encountered by earlier investigators was the failure to reach a single concentration level when starting with supersaturated solutions or with undersaturated solutions. Levinskas and Neuman[25] were able to show that this anomaly was an artifact and obtained substantially identical final concentrations whether the solutions were supersaturated or undersaturated initially. Levinskas and Neuman[25] removed suspended solids, selected the nature of the solid, maintained a constant pH, controlled the presence of bicarbonate, and precisely fixed the relative amounts of solid to solution phases. They were thereby able to select conditions in which the surface composition of the synthetic apatite did not change appreciably during equilibration.

Difficulties of the solubility studies. There is a critical relation between the amount of solids shaken with a given volume of solution and the amount of material that goes into solution. Neuman and Neuman recently summarized the situation as follows: "In general, the amount of material dissolved is found

to increase with increasing solid phase."[23] Numerically, the calcium concentration increases in such a way that, when the amount of solid is increased 1000 times, the calcium concentration increases 10 times.

The concentration of calcium in solution increases with the concentration of sodium in the solution. This phenomenon, designated as heteroionic exchange by Neuman *et al.*,[24] involves the replacement of calcium ions in the crystal surfaces by sodium from the solution. Levinskas pointed out that, because this exchange reaction occurs, the solubility of bone or of synthetic apatite cannot be studied under conditions similar to the relation *in vivo*, because plasma and extracellular fluid contain sodium.[23]

The interpretation of analytical data on solutions equilibrated with bone or synthetic hydroxylapatite is greatly complicated by the fact that hydrolysis occurs. The basic calcium phosphates do not dissolve in molecular formula ratios.

Summary. No solubility product has ever been demonstrated for hydroxylapatite, the mineral of the hard tissues, and, by virtue of the variable composition of the hydroxylapatite crystals as a result of an indefinite series of isomorphous substitutions, no solubility product can be calculated.

In clinical practice, the Howland-Kramer product (total calcium times inorganic phosphate of serum) is a reliable index of discrimination between active rickets and healing or no rickets. Although this product has a superficial resemblance to the $K_{s.p.}$ $CaHPO_4$, the secondary salt is not deposited in the body and probably plays no role in calcification or in skeletal metabolism. It is an interesting and unexplained phenomenon that the calcium and phosphorus concentrations in solutions in contact with $CaHPO_4$ and with hydroxylapatite are related empirically as follows: the logarithm of the calcium concentration is a linear function of the logarithm of the phosphate concentration minus pH. By an efficient removal of suspended solids, the equilibrium condition in solutions in contact with synthetic hydroxylapatite has been reached promptly and reproducibly whether the solution was initially supersaturated or undersaturated. Calcium concentration in solution increases with the amount of solid phase, and with the concentration of sodium in solution. Although bone salts must be in moment-to-moment equilibrium with the circulating and extracellular fluids, this relation has not been adequately characterized.

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RADIOTRACER STUDIES OF HARD TISSUES*

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One of the first of the important discoveries made with artificial radiotracers, soon after radiophosphorus became available, was the demonstration of the unexpectedly large and rapid incorporation of this radioisotope in calcified tissues. These results, and those of subsequent studies with radioisotopes, have shown that the skeleton and the teeth are not static structures but are dynamic tissues in which the elements are constantly turned over and renewed. The revolution in our concept of the physiology of the calcified tissues which was made necessary by the work with radioisotopes is well described by an extract from an address given by Professor August Krogh, on Sept. 10, 1936, at the Harvard Tercentenary Celebration.[1] In this address, Professor Krogh referred briefly to the early work of Hevesy with radiophosphorus, then just beginning to be published,[2, 3, 4] and said:

"To my mind the most interesting result is the extensive exchange taking place in bones and teeth. It is, of course, well known that the organism is able to draw upon the skeletal system as a reserve of inorganic salts but, even remembering this, I have never before been able to look upon the atoms deposited in practically insoluble salts and at a considerable distance from blood vessels, in the dentine for instance, as being in constant interchange with the atoms of the salts in tissue fluids and blood. This is, however, what the experiments clearly indicate."

The extent and the rapidity of skeletal mineral turnover, demonstrated with radioisotopes, are such as to indicate clearly that the inorganic ions of calcified tissues are intimately associated with all other aspects of the transport and metabolism of these ions. An example of a research which is frequently cited to indicate the high order of rate of turnover of skeletal phosphorus is the work reported by Hevesy, Levi, and Rebbe.[5] These workers maintained a constant specific activity of plasma inorganic phosphorus of rabbits over a period of several weeks by spacing of repeated intravenous injections of radiophosphorus as inorganic phosphate. The specific activity of the bone phosphorus as a percentage of the specific activity of the plasma inorganic phosphorus gave the results in TABLE 1, which denote the fractions of the phosphorus in the bones which were renewed in 50 days. For example, about one fourth of the phosphorus of the femur and tibia epiphyses was replaced in 50 days. Smaller fractions of the phosphorus of long bone diaphyses were replaced but, even in this case, the quantity of phosphorus involved is considerable.

The rate of turnover of skeletal calcium and sodium can be estimated in the living animal without the necessity of maintaining the plasma specific activity constant by following the rate and degree of dilution in the blood of intravenously injected radioisotopes of these ions. This procedure is applicable only to those inorganic ions which are located solely or mainly in bone mineral and

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TABLE 1
EXTENT OF REJUVENATION OF RABBIT BONES

In 50 days	Phosphorus replaced %
Femur epiphysis	29.7
Femur diaphysis	6.7
Tibia epiphysis	28.6
Tibia diaphysis	7.6
Ribs	27.5
Scapula	43.8
Incisor dentin (root)	103.0

extracellular fluid. This method, therefore, cannot be used to measure the rate of turnover of skeletal phosphorus or potassium.

In our experiments[6] dogs received a rapid intravenous injection of a solution containing both calcium[45] and sodium[22]. Arterial blood samples were obtained, beginning at one minute after the injection and at frequent intervals thereafter, from a cannula which had been inserted into the femoral artery. The concentration of each of the isotopes in the arterial plasma samples was determined and the results are shown by the points on the curves in FIGURE 1. The concentration of the radioisotopes in the plasma fell rapidly; that of calcium more rapidly than that of sodium, and the concentration of radiocalcium continued to fall long after an equilibrium concentration of radiosodium in the plasma had been reached. Obviously, equal numbers of the ions must move simultaneously in both directions between adjacent compartments since the concentration of each of the ions in plasma and interstitial fluid is held constant. The concentrations of the radioisotopes in the plasma fell because of the movement of the labeled ions from plasma to interstitial fluid and from interstitial fluid to bone. The labeled ions in the fluid compartments were thus replaced with a similar number of unlabeled ions.

The radiocalcium and radiosodium in the plasma rapidly became diluted in quantities of calcium and sodium which far exceeded the amounts of these ions present in the extracellular fluid compartments. By use of a modification of the isotope dilution equation, these data were used to estimate the quantities of skeletal calcium and sodium exchanged at each of the time intervals. It is assumed upon sound grounds that bone is the only tissue which can supply significant quantities of calcium or sodium ions to extracellular fluid.

In the equation as used (FIGURE 2), the first step was to calculate the total quantity of sodium or calcium through which the injected isotopes were distributed in order to give the observed activity per milligram of each element in the extracellular fluid as sampled by the plasma. The first two terms in the equation gave this result. From this quantity was subtracted the amount of calcium or sodium in extracellular fluid to give the amounts of these elements which had been exchanged by bone at each of the time intervals after the injection.

This equation requires that it be assumed that the specific activity of each labeled ion is equal in plasma and in interstitial fluid at any given time. This assumption is incorrect over the very early minutes after the injection, but the

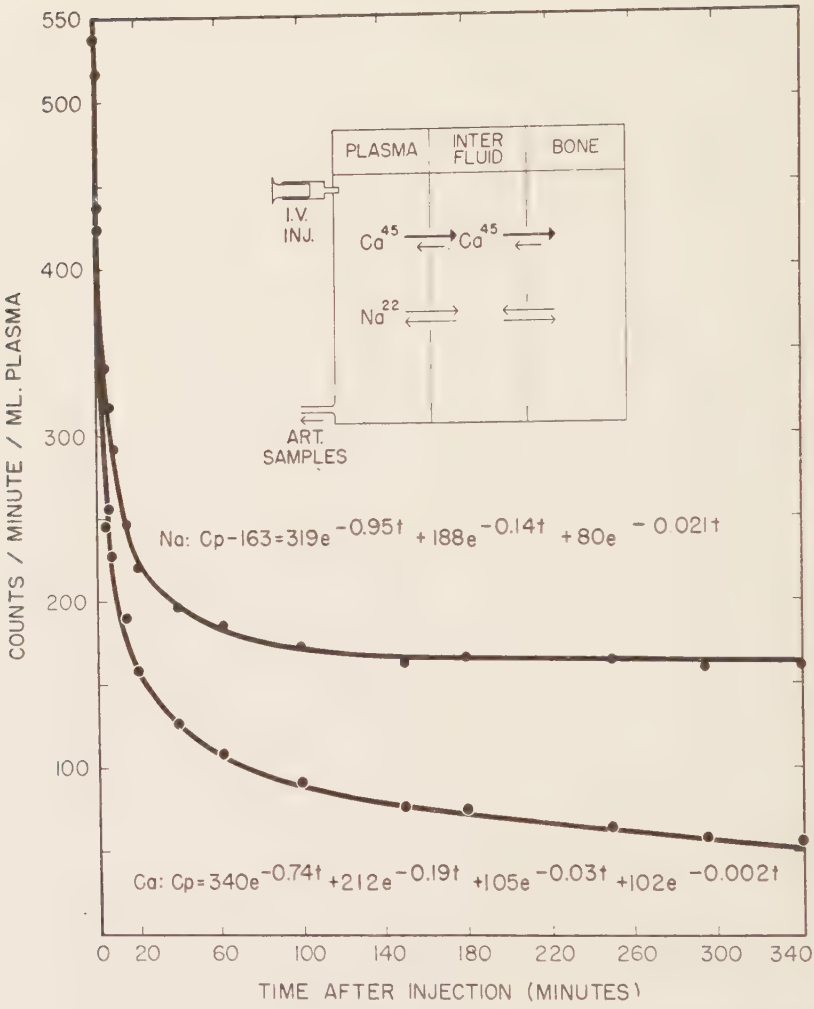

FIGURE 1. Arterial plasma concentrations of Na^{22} and Ca^{45} following the intravenous injection of these radioisotopes. A diagrammatic illustration of the experiment and of the paths of movement of the labeled ions between plasma, interstitial fluid, and bone is given.

error thereby introduced rapidly decreases with time. Certainly, by 150 minutes, the calculated quantities of exchanged bone calcium and sodium are liable to only a small error.

The results show (FIGURE 2) that, by 150 minutes, the skeleton of a 20-kilogram dog had exchanged about 1 gm. of calcium and about 8.2 gm. of sodium. Both of these quantities are large and indicate that significant amounts of bone mineral calcium and sodium are delivered to the fluid compartments of the body in short periods of time. The quantity of sodium is of the order of half of the entire amount present in the skeleton. The very rapid turnover of skeletal sodium, compared to the slower rate of turnover of skeletal

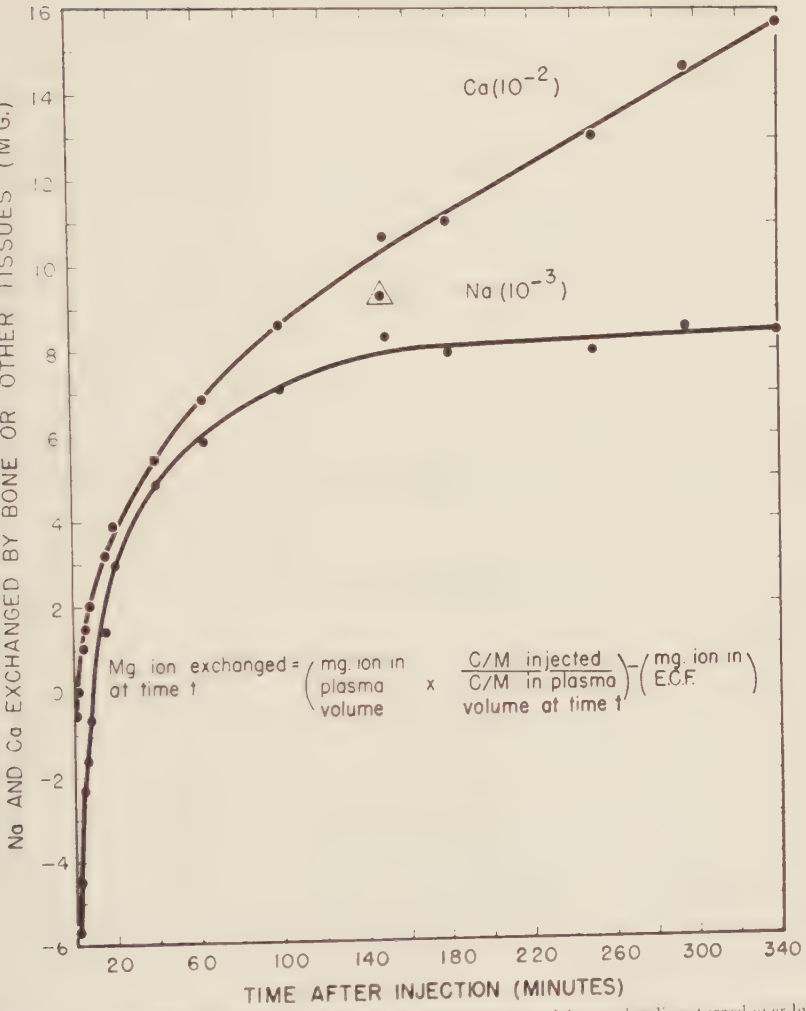

FIGURE 2. Isotope dilution equation used to calculate quantities of calcium and sodium turned over by the skeleton and the results of these calculations.

calcium, is consistent with the Hendricks-Hill[7] description of the differences in location of sodium and calcium in the apatite crystal.

It is not correct to conclude that bone mineral is dissolved, removed, and replaced with newly deposited bone mineral, through the operation of the biological processes of bone resorption and bone formation in order to account for the degree of turnover of the bone mineral which is demonstrated by the accumulation of radioisotopes in the skeleton. The mechanism in largest part is one in which the labeled ions, for example phosphate, in the body fluids exchange and replace an equal number of unlabeled ions in the bone salt. This concept was legitimately drawn by inference from the whole animal experi-

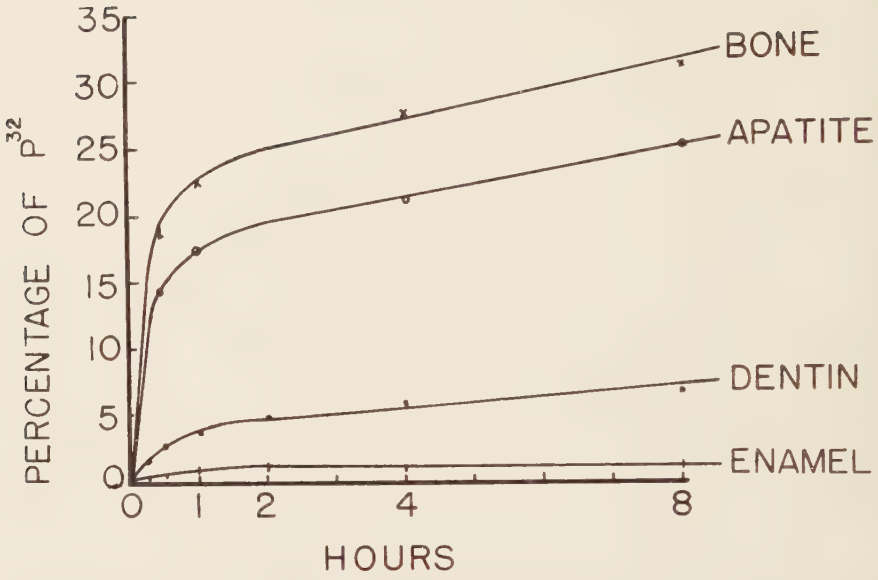

FIGURE 3. *In vitro* exchange of phosphate by mineral phases of bone, enamel and dentin, and apatite for labeled phosphate in solution (Falkenheim, Neuman, and Hodge[8]).

ments[2, 3, 4] but the direct evidence for this conclusion was first obtained from experiments carried out *in vitro*. It was shown[5] that dead calcified tissues rapidly remove large amounts of radiophosphorus from solution without affecting the total concentration of phosphate ion in the solution.

The characteristics of the *in vitro* accumulation of phosphate by powdered calcified tissues and by synthetic apatite are well shown by FIGURE 3, taken from the work of Falkenheim, Neuman, and Hodge.[8] The radiophosphorus was, at the start of the experiments, added to the solution phases, and the quantities of the radioisotope present in the insoluble calcium phosphates were determined at various times. Since the labeled phosphate ions in these experiments were acquired by the solid phases without loss of phosphate by the solution, an exchange of equal numbers of phosphate ions between the insoluble materials and the solutions was demonstrated.

It is seen that the rate of exchange of phosphate ions is initially rapid, and that the rate falls off with time. By comparison of the specific activities of the phosphorus of the solid and aqueous phases, it was shown[8] that about 12 per cent of the phosphorus of bone inorganic material was exchanged with a 2×10^{-3} molar phosphate solution in eight hours. In experiments of longer duration, about 19 per cent of phosphorus was exchanged in 10 days. Comparable quantitative results were obtained by Falkenheim, Underwood, and Hodge[9] for calcium exchange by bone inorganic material in *in vitro* studies employing radioactive calcium.

Lindenbaum,[10] with the writer, has examined the *in vitro* exchange of calcium and phosphorus of apatite for ions in solution in the reverse direction by follow-

ing the movement of labeled ions from apatite to solution. A synthetic hydroxyapatite which was doubly labeled with radiocalcium and with radiophosphorus was used. In separate experiments, this doubly labeled apatite was exposed to solutions of inactive calcium ion or inactive phosphate ion, each at three concentrations (FIGURE 4). From the radioactivity assays of the bathing solutions, after 100 hours of incubation at 37° C., the quantities of calcium or phosphate transferred from the solid phases to the solutions were determined. In FIGURE 4, the logarithms of the quantities of calcium or phosphorus transferred from apatite to solution per milligram of apatite are plotted against the logarithms of the calcium or phosphate ion concentrations of the bathing solutions. It will be noted that the results do not fit the empirical Freundlich adsorption isotherm equation, as was found when the reverse process, that is uptake of radioisotopes of phosphorus,[11] sodium,[12] and fluoride[13] from solutions to the solid phase, was studied.

The quantities of calcium and phosphate transferred from the apatite to the solution were increased as the calcium and phosphate concentrations of the bathing solutions were increased. The two lower ion concentrations bound the accepted normal calcium and phosphate ion concentrations in interstitial fluid. At these two ion concentrations, two times as much apatite calcium as apatite phosphorus was exchanged, which happens to coincide with the relative amounts of calcium and phosphorus in apatite. These results suggest that the

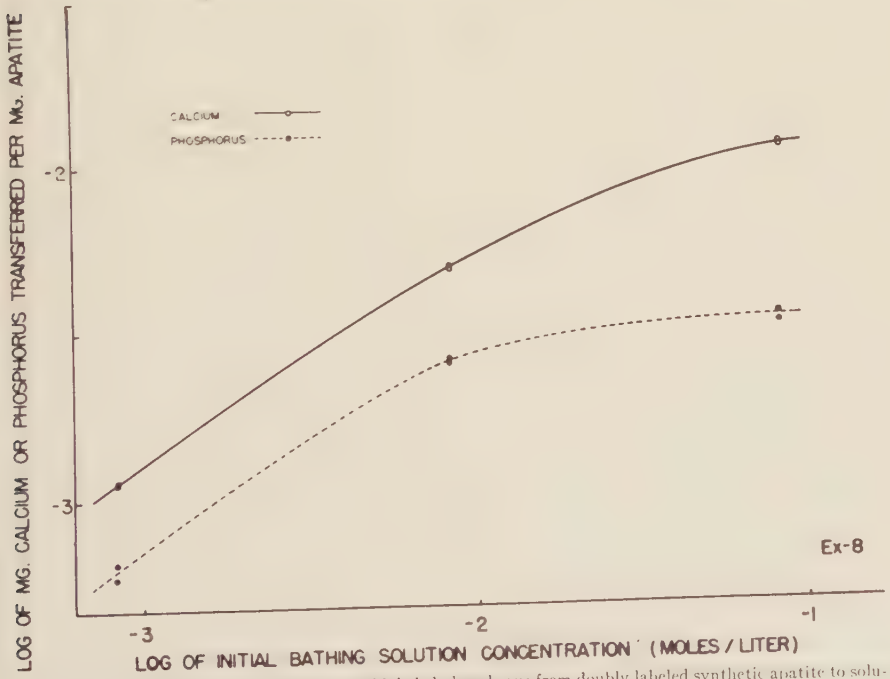

FIGURE 4. Transfer of labeled calcium and labeled phosphorus from doubly labeled synthetic apatite to solutions of inert calcium and phosphate (Lindenbaum[10]).

apatite molecule is turned over as a unit in solutions of physiological concentrations of calcium and phosphate ions. However, at the highest ion concentrations in the bathing solution, about three times as much calcium as phosphorus was exchanged. This finding is evidence that the calcium and phosphorus of the apatite molecule can exchange independently.

It should be noted that smaller fractions of the apatite calcium and phosphorus were exchanged in these experiments than were found in the studies of the uptake of radiophosphorus and radiocalcium by apatite from solution. For example, in these experiments, at the highest ion concentration, 2 per cent of the apatite phosphorus was renewed in 100 hours, with correspondingly lower amounts at the lower ion concentrations. In the work of Falkenheim, Neuman, and Hodge,[8] 7.6 per cent of the phosphorus of a synthetic apatite was exchanged in eight hours using a 2×10^{-3} molar phosphate solution. It will require further consideration to understand why the results employing an apatite in which the calcium and phosphorus were uniformly labeled with the tracer isotopes, gave lower results as to fraction of the mineral ions turned over, than was obtained in experiments in which the movement of ions from solution to solid phase was followed with radiotracers. Nevertheless, the new results confirm the exchange hypothesis and furnish another item of data as to the rate of the process.

The rapid exchange of inorganic ions between body fluids and bone mineral can be seen to afford the basis of an important mechanism for precise and speedy control of the electrolyte composition of body fluids, since exchange of ions would facilitate rapid net shifts in both directions of the ions between bone mineral and body fluids. The rapid circulation of large amounts of these ions between bone mineral and body fluids indicates a physiological unity of the skeleton and a unity of the skeleton with the organism as a whole. It can be indicated that the rapid turnover of calcium between the skeleton and body fluids is a process which permits quick and competent support of calcium ion concentration in the body fluids in the absence of calcium ingestion. The skeleton as a whole is accordingly able to supply calcium and also phosphate ions to sites of calcification, as for example to a fracture site.

Another constituent of bone mineral which exhibits a rapid rate of turnover is the bone inorganic carbon. Our studies[14] show that the biological half-life of bone inorganic carbon in growing rats is 12 to 15 days and, in mature rats, about 30 to 40 days.

A variety of methods have been used to administer radioactive carbon dioxide to animals and to build up and maintain the radiocarbon label of body fluid inorganic carbon. In one phase of our work,[15] this result was obtained by the continuous intraperitoneal injection of C^{14} labeled sodium carbonate. In one experiment, a large rat received sodium carbonate in this manner by injection over a period of 116 hours. When this animal was sacrificed, radiocarbon was found in every tissue examined. The skeleton as a whole had accumulated about 0.1 per cent of the injected radiocarbon (TABLE 2). Since the specific activity of the bone inorganic carbon was higher than that of any other tissue carbon, this carbon had the highest rate of turnover of any fixed carbon in the body. The incisor teeth inorganic carbon is a special case, due to the fact that

TABLE 2

DISTRIBUTION IN TISSUES AND TISSUE COMPONENTS OF RADIOACTIVE CARBON FOLLOWING CONTINUOUS INTRAPERITONEAL INJECTION AS SODIUM CARBONATE

Tissue or tissue component	Fraction of total dose per cent	Specific activity $\times 10^6$	Relative specific activity (bone norg. carbon = 100)
Bone inorg. C		715.4 ± 6.25	100.0
Bone	0.1162 ± 0.0006	90.0 ± 0.46	12.6
Bone protein		25.5 ± 0.41	3.6
Incisor teeth	0.0016 ± 0.00002	76.9 ± 0.82	10.7
Incisor teeth inorg. C		1138.9 ± 5.3	159.2
Molar teeth	0.0008 ± 0.00001	51.5 ± 0.68	7.2

radiocarbon was accumulated by deposition consequent upon growth of the teeth as well as by exchange.

In 1949, Lindenbaum[10] and the writer carried out some studies of the *in vitro* transfer of radiocarbon from solution to bone mineral, with the notion that such studies would contribute information on the problem of the location of inorganic carbon in the apatite crystal. Some of the observations and results of this study may now be of interest in relation to the Hendricks-Hill[7] description of the crystal surface location of apatite inorganic carbon.

The uptake of radiocarbon as carbonate by bone inorganic material from a 0.066 molar sodium carbonate solution labeled with C^{14}, from 1 to 240 hours at 37° C., is shown by FIGURE 5. The results are given as milligrams of carbon

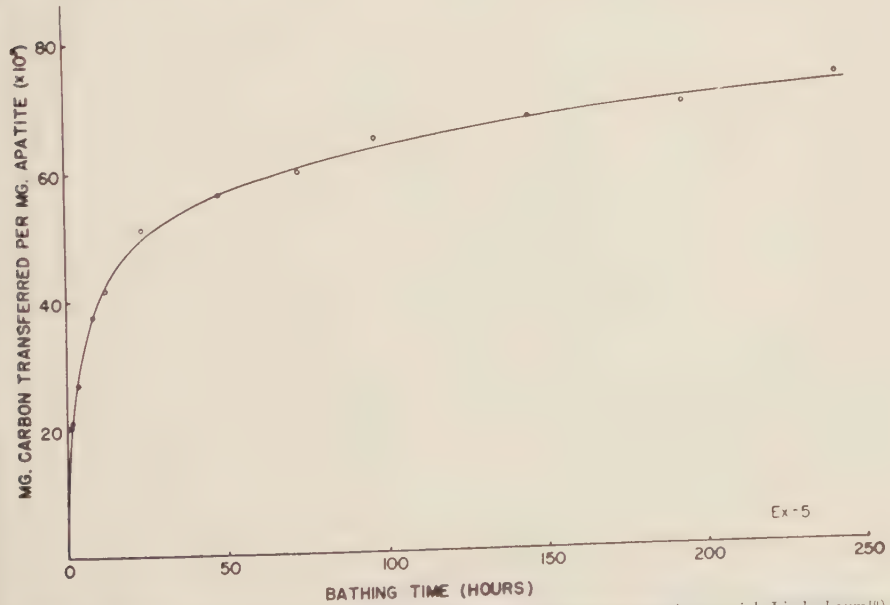

FIGURE 5. Transfer of radiocarbon labeled carbonate from solution to bone inorganic material (Lindenbaum[10])

transferred from solution per milligram of bone material. The bone inorganic material used was the glycol-KOH ash of beef bone. The uptake of carbonate from solution was very rapid over the first few hours and, after the hundredth hour, the rate of uptake of the labeled carbon became very slow. At the end of the experiment, about 8 per cent of the inorganic carbon had been replaced. These results were obtained by calculation from the data of measurements of the radiocarbon found on the solid phases after the end of the incubation periods. For reasons to be explained below, the results of radiocarbon uptake, and hence for carbon transferred from solution to solid phases determined in this way, may be somewhat too low. Nevertheless, it appears that the concept of crystal surface location of inorganic carbon in apatite would lead to the expectation that very much larger fractions of the bone inorganic carbon should be exchanged than were found in these experiments.

The quantity of carbonate transferred to the bone mineral in 100 hours from solution was increased, as the concentration of carbonate, expressed in FIGURE 6 as milligrams of carbon per milliliter of bathing solution, was increased. It is seen that, except for the highest concentration of sodium carbonate, a general linear relationship of the logarithmic plot was obtained.

Unlike the results obtained with radioisotopes of phosphorus and calcium, there is some evidence that processes other than those of exchange are involved as a part of the mechanism responsible for transfer of carbonate from the bathing solution to the solid phase. First, it was found the total carbon content of the solutions decreased and the total carbon content of the solid phases increased in some of Lindenbaum's experiments. Of even greater significance in this regard is the agreement in quantities of sodium and carbonate removed

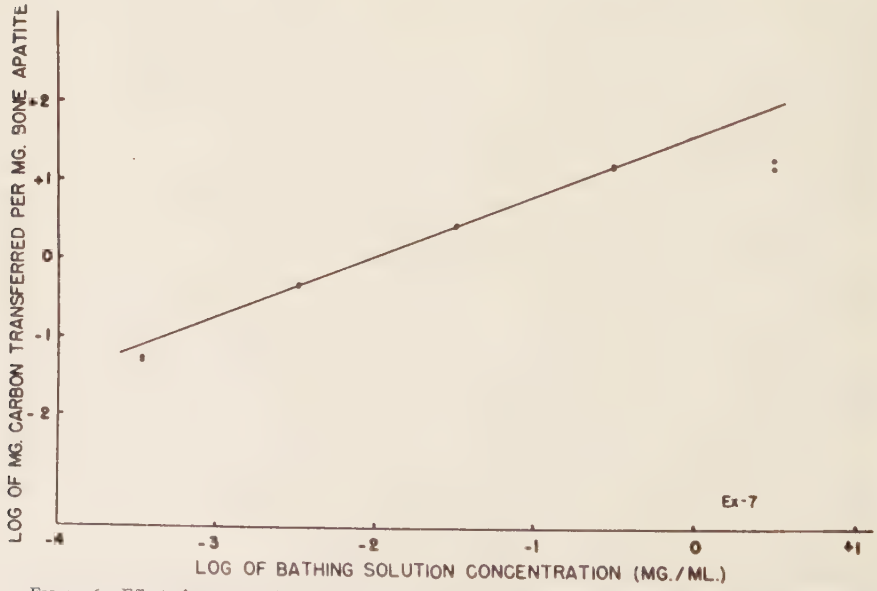

FIGURE 6. Effect of concentration of carbonate in solution on transfer of labeled carbonate from solution to bone inorganic material (Lindenbaum[10]).

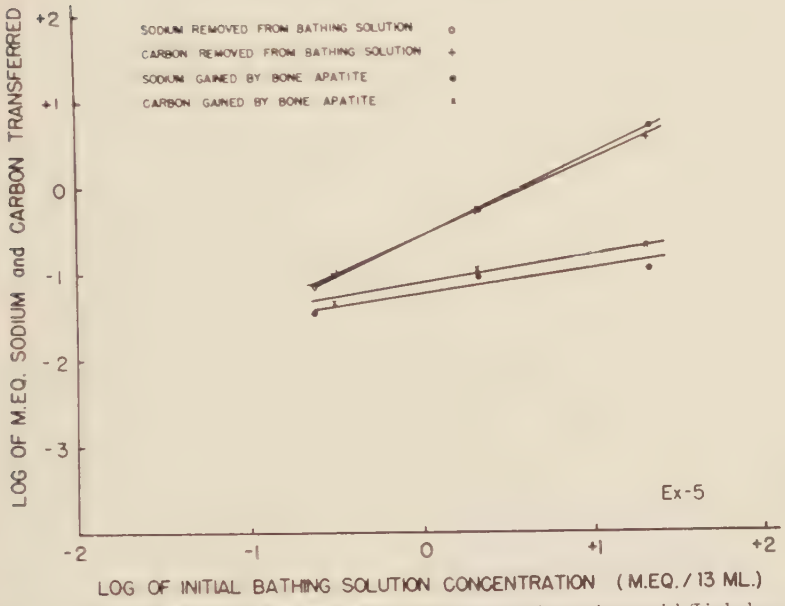

FIGURE 7. Transfer of sodium and carbonate from solution to bone inorganic material (Lindenbaum[10])

from solutions of sodium carbonate by bone inorganic material. The data which gave the results plotted as the upper pair of nearly congruent lines in FIGURE 7 were obtained from the sodium and carbonate analyses of the bathing solutions after 100 hours of incubation with bone inorganic material. These results indicate that nearly identical equivalent quantities of sodium and carbonate were removed from the aqueous phases to the solid material at the three initial concentrations of sodium carbonate shown.* This finding suggests that the ions equivalent to the entire sodium carbonate molecule are transferred from the solutions to the surface of the micro crystals of the apatite.

The lower two parallel lines in FIGURE 7 give the results of quantities of sodium and carbon gained by the bone mineral as obtained by chemical analysis and radioactivity assay of the bone mineral specimens recovered from the bathing solutions. These results are lower than those obtained by examination of the solutions, whereas, in fact, the result obtained by the two methods should be in agreement. The lower results for the sodium and carbon transferred, obtained by examination of the solid phase, are now believed to be due, in large part, to loss of lightly held radiocarbon and sodium from the solid phases, when these were washed after the solids were recovered from the bathing solutions.

The very loose attachment of some carbon to bone mineral apatite is also shown by the results in FIGURE 8. Two duplicate samples of bone mineral were

* Solutions with a very low initial total sodium concentration (approximately 4×10^{-3} milliequivalents per 13 ml.) showed an actual gain of sodium after incubation with the bone inorganic material. This result would imply solution of sodium from the solid phase, but the accuracy of the result is affected by error of analytical determination of sodium at the very low concentrations.

labeled with radiocarbon by exposure to a solution of sodium carbonate containing C^{14}. These samples were dried and placed in counting dishes and stored in a dessicator over phosphorus pentoxide. The samples were removed from the dessicator and counted at intervals over a period of 42 days. The results show that there was a very rapid loss of activity over the first 4 to 5 days and that there was a further slower loss of activity up to at least 42 days. It is thus demonstrated that radiocarbon may be removed from bone mineral on storage. These observations, and those having to do with the loss of C^{14} inorganic carbon from bone by washing, point out the hazard of quantitative interpretations of radiocarbon transfer to bone mineral based upon radioactive assay of the mineral itself. Such experiments are best carried out by examination of the bathing liquid.

It is clear that these results show that at least some carbon of bone mineral has a crystal location which gives it a very frail attachment and, therefore, cannot be situated in the lattice proper.

Our knowledge of the physiology of the teeth has been notably advanced through studies with radiotracers. The old question of the permeability of enamel and dentin has been answered by the demonstration of a two-way transport of labeled ions and of some molecules through these dental hard tissues.

When radioisotopes of phosphorus and calcium are given to experimental animals by injection or by stomach tube[16] these isotopes can be found in the enamel and dentin (TABLE 3). Dentin acquires much smaller amounts of these mineral isotopes than bone, and the specific activity of enamel is, in different experiments, of the order of 5 to 20 per cent that of dentin. Whether the

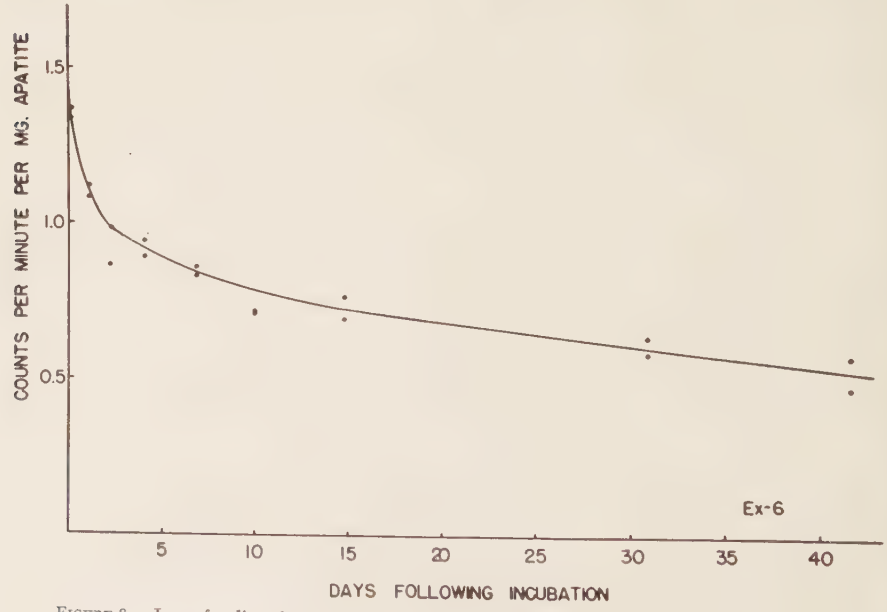

FIGURE 8. Loss of radiocarbon activity from bone inorganic material on storage (Lindenbaum[10])

TABLE 3

RADIOCALCIUM AND RADIOPHOSPHORUS UPTAKE BY CALCIFIED TISSUES

(Isotopes given simultaneously by stomach tube five days prior to sacrifice of the rat)

	Radio Ca (rel. spec. act.)	Radio P (rel. spec. act.)
Femur epiphyses	92	80
Femur diaphyses	82	75
Incisor teeth dentin	55	52
Incisor teeth enamel	27	27
Molar teeth dentin	12	14
Molar teeth enamel	1.2	2.7

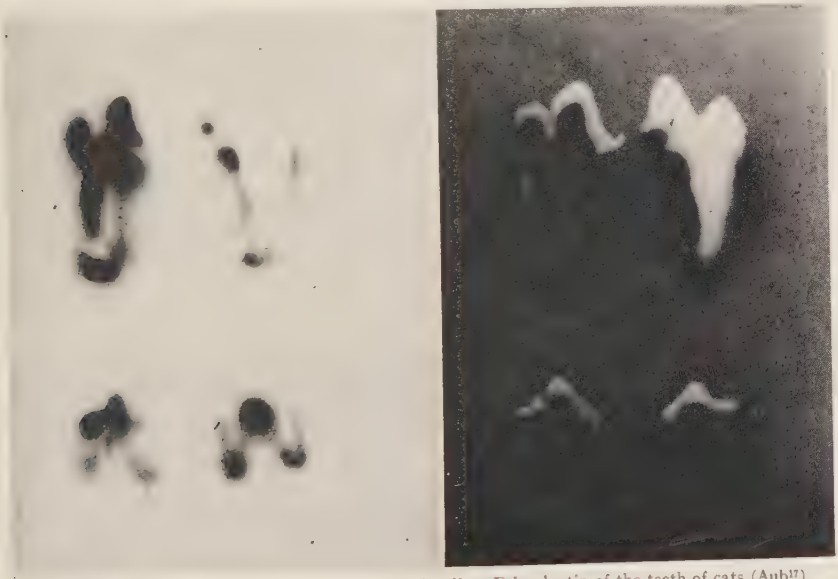

FIGURE 9. Radioautographs showing uptake of radium-D by dentin of the teeth of cats (Aub[17])

avenue of entrance of labeled isotopes into the enamel is from the blood via the dentin or from the saliva has been debated. The best evidence is that both avenues are open and operating. In the case of radiocalcium and radiophosphorus, it appears that the transport from saliva to enamel is relatively the more important.

FIGURE 9 is of interesting historical significance. It is probably the first clear demonstration of the uptake of a radioisotope by the dentin of nongrowing teeth. It represents, therefore, the first evidence that the dentin is accessible to the mineral ions in the body fluids. Doctor Joseph Aub showed this slide, which is obviously that of radioautographs of ground sections of teeth (the teeth are those of cats), in April 1938, at a Bone Symposium at the Baltimore meeting of the American Society of Biological Chemists. The radioisotope is radium-D, which is isotopic with lead, the isotope having been given to the animals by injection. The radioautographs show the greater accumulation

FIGURE 10. Radioautograph showing diffusion of radiocarbon labeled urea into enamel of a human tooth from the surface of the tooth (Wainwright and Lemoine[18]).

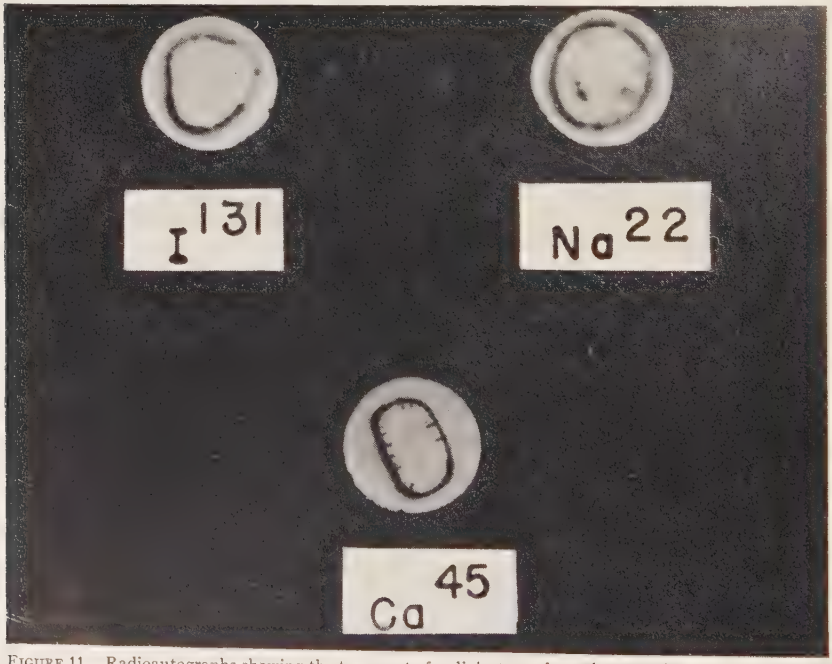

FIGURE 11. Radioautographs showing the transport of radioisotopes from the enamel surface into the enamel of human teeth.

of the radiolead in the dentin than in the enamel. I am informed by Doctor Aub that this work should appear in print within the next few months.[17]

The centripetal migration of radioactive ions and labeled molecules applied to the surface of the enamel towards the dentin has been observed both in the living animal and with extracted teeth. Wainwright used extracted human teeth in *in vitro* experiments to show the diffusion of C^{14} and S^{35} labeled compounds into the enamel. FIGURE 10 is a radioautograph of a tranverse section through the crown of a tooth which had been moistened over the enamel surface with C^{14} labeled urea solution for five minutes.[18] The diffuse penetration of the labeled urea to the dentoenamel junction in many areas is obvious.

The radioautographs in FIGURE 11 are our own and were prepared from human teeth whose crowns had been immersed in the solutions of the indicated radioisotopes. The migration of these ions into the enamel is evident. These and other studies of the centripetal migration of ions which are part of the enamel mineral indicate that exchange of ions between the enamel and saliva is a normal physiological process.

Perhaps the most dramatic and remarkable demonstration of centripetal transfer of ions from the surface of the teeth has been seen with radioiodine. In the studies of Bartelstone,[18] the tips of the upper canine teeth of living cats were immersed in a solution of iodide labeled with I^{131}. The radioiodide was absorbed through the enamel and dentin and reached the circulation, as was made evident by the accumulation of the radioisotope in the thyroid gland.

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RECRYSTALLIZATION IN BONE MINERAL*

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Through the years, one of the most puzzling aspects of bone metabolism has been the apparent irreversibility of isotope-fixation by the skeleton. Within minutes after an injection of isotopic calcium or phosphate the SA (specific activity in counts/mg.) of the bone rises rapidly but, as the SA of the blood continues to fall, the SA of the bone mineral remains almost constant over protracted periods.[1] Ionic exchange reactions occurring on the surfaces of the mineral crystals of bone can account for the rapid phase of the isotope incorporation.[1] Such a surface-exchange reaction is readily reversible, however, and the SA of bone should fall in a parallel fashion with the declining SA of blood, were surface-ionic exchange the only process involved.[1, 2] It is true that the growth process, through the formation of new mineral, can and does "bury" radiocalcium and radiophosphate irreversibly, but growth alone cannot account for the magnitude of the fixation process,[3] especially in experiments with adult animals.

A few years ago, experiments *in vitro* demonstrated that ion exchange was not limited to the *surfaces* of the mineral crystals, but that ions from crystal interiors can also equilibrate with the fluid medium.[2] This process, termed "recrystallization," appeared to be irreversible, was temperature dependent, and required the presence of an aqueous phase.[2, 4] Conceivably, such a process could account for the puzzling irreversibility of isotope-fixation that troubled investigators for years. Subsequently, data were presented which indicated that recrystallization does, indeed, take place in the living animal,[5] and the concept has proved especially useful in explaining isotope-distribution experiments in nongrowing animals.[6] As will be shown, the following processes: surface exchange; recrystallization; and growth (with attendant remodeling), adequately explain the rapid fixation and slow release of isotopes of calcium and phosphate by the skeletal mineral.

The actual *mechanism* of the recrystallization process and its quantitative significance *in vivo* have remained obscure, however. Just how does the crystal interior equilibrate with ions in the fluid medium? How much of the skeletal mineral is readily exchangeable? How much of the total skeletal mineral recrystallizes?

To obtain partial answers to these questions, two types of experiments have been performed. Physicochemical studies of the interaction of hydroxyapatite crystals and buffer solutions were designed to elucidate the reaction mechanism,[7, 8] and a few selected studies of adult rats given radiocalcium in the diet were carefully arranged to give information bearing on quantitative relations *in vivo*.

* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

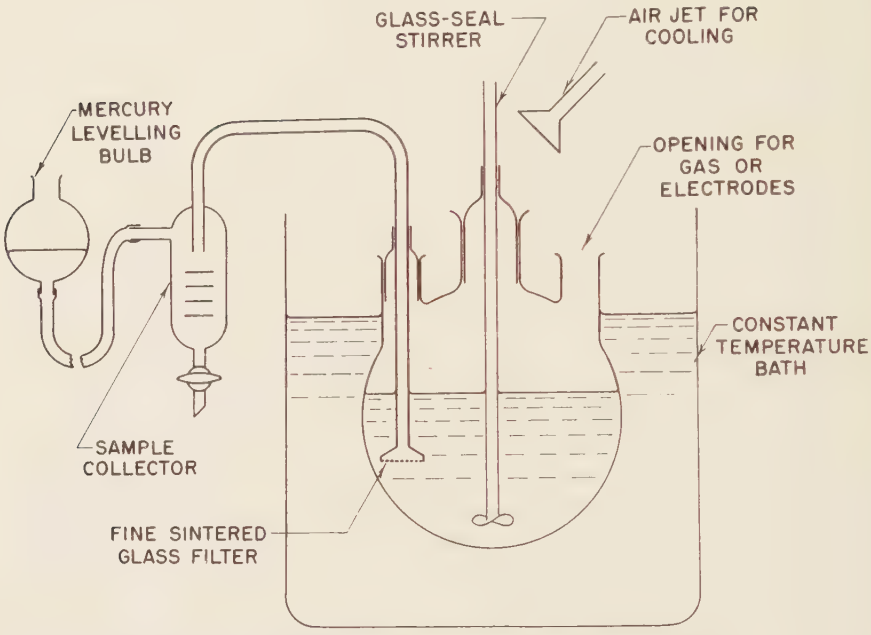

FIGURE 1. The apparatus used in the kinetic study of the exchange process

On the Mechanism of Ionic Exchange

To study the exchange phenomena, the apparatus described by Schweitzer and Nehls[9] was employed. This apparatus is illustrated in FIGURE 1. A known weight of apatite crystals is placed in the flask (usually 1 gm.) and, after the addition of water or buffer (usually 700 ml.), stirring is commenced. When the suspension has reached a solubility equilibrium (in this case 18 hrs. was sufficient),[10] a small aliquot (less than 1 ml.) containing the radioisotope is added. At various time intervals thereafter, one ml. samples of crystal-free solution are removed for radioactivity assay. At termination, a large sample is taken for analysis of calcium,[11] phosphate,[12] pH, *etc.* Note that, under these circumstances, the large volume of solution and the small sampling volume insure that equilibrium conditions are not significantly altered during the experiment. In this way, it is possible to obtain data on the effect of variables on the rate of disappearance of isotope from solution or, conversely, the rate of incorporation of isotope by the solid.

In this instance, where many variables are inextricably interlocked, the problems of designing experiments under nonequilibrium conditions are forbiddingly difficult. Therefore, only the kinetic approach was attempted even though much useful information on the equilibrium itself could not be obtained; specifically, such useful constants as ΔF and ΔS.

A typical curve for the disappearance of isotopic phosphate from the solution is given in FIGURE 2. The P^{32} concentration has been expressed in logarithmic terms; therefore, the linearity of the several portions of the curve indicate a

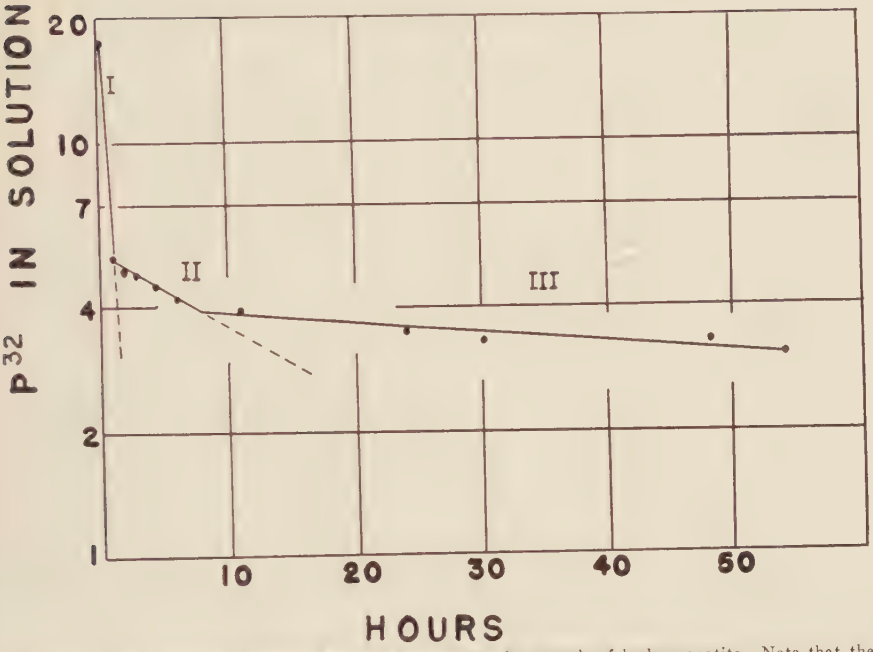

FIGURE 2. The removal of labeled phosphate from solution by crystals of hydroxyapatite. Note that the process occurs in a series of first-order steps designated I, II, and III.

series of first order reactions varying in rate from very fast to very slow. The rate constants, in reciprocal hours, can be obtained directly from the slopes of the linear portions of the disappearance curve. Other conditions being constant, these rate constants were independent of stirring and, therefore, have real physical meaning. Of the series of reactions, only those labeled Steps II and III, FIGURE 2, were studied. Step I was too fast to be measured accurately, and Steps IV *et seq.* were too slow and variable. A large series of such disappearance curves was obtained under a variety of conditions. From the curves, the rate constants for Steps II and III were obtained and subjected to kinetic analysis. This analysis has provided data from which certain inferences may be drawn concerning the actual reaction mechanics.[8] These inferences will be briefly reviewed.

To understand the mechanics, however, it is necessary to consider first the physical state of the crystal; *i.e.*, solution interface[13, 14] as a frame of reference. This interface is presented diagrammatically in FIGURE 3, where it can be seen that an ion may occupy a number of different positions: in the bulk solution, in the hydration shell,[14] in the crystal surface, and at various levels within the crystal lattice itself. The most obvious assignment of reaction rates to specific reaction sites is to attribute the fast reaction, Step I, to the exchange of phosphate ions in the bulk solution with ions in the hydration shell. Step II then becomes the interchange of ions from the hydration shell with ions in the crystal surface, and Step III represents an interchange of surface ions with subsurface ions.

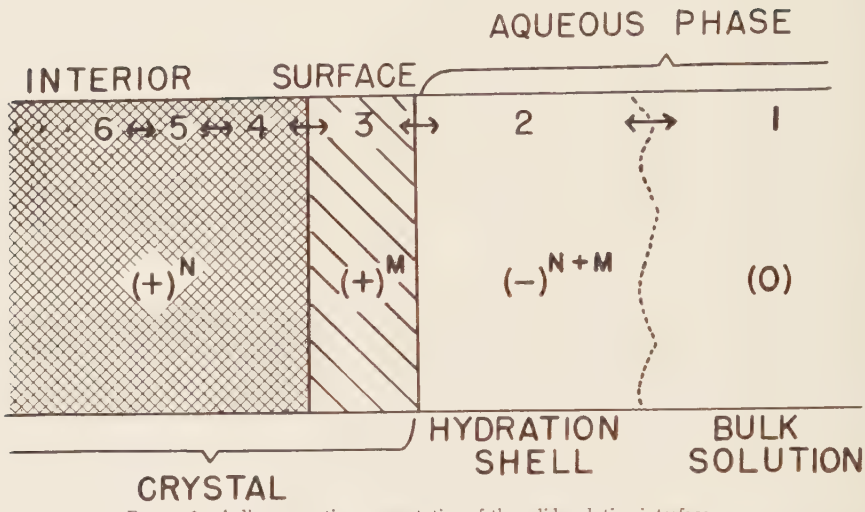

FIGURE 3. A diagrammatic representation of the solid: solution interface

This assignment is supported by the fact that increasing the ionic strength of the solution reduces the rate constant of Step II, the surface reaction, but does not affect the rate constant of Step III, the thermal interchange of ions *within* the crystal.[15] These data are presented in FIGURE 4.

Since these rates were measured under equilibrium conditions, there is no justification *a priori* for assuming the rate-determining step to be the transfer of labeled ions from solution to solid. It is just as likely that the transfer of nonisotopic ions from solid to solution might be rate-determining. Actually, the kinetic data assembled in TABLE 1 strongly suggest that the rate-determining step is the thermal ejection of surface ions into the hydration shell. Note that the values for both energy of activation and entropy of activation for Step II are similar in sign and magnitude to those for Step III, which *must* be a thermal process occurring within the crystal. Such a mechanism, unlike a collision mechanism, is consistent with the lack of effect of phosphate concentration on reaction rate. Finally, the effect of ionic strength is best explained in terms of the "thermal escape" mechanism. Since the crystals are positively charged[14] and possess, therefore, a surrounding field of anions, the escape of negative phosphate groups must be impeded by a potential barrier. Since increasing ionic strength increases the charge on the crystal[7, 8] and therefore increases the potential barrier, a reduced rate is to be expected.

In terms of this new information, what is "recrystallization"?

It can be concluded that the recrystallization process does not involve a solution and redeposition of ions, as some have erroneously inferred. It is rather an extension of the surface exchange reaction, mediated by the thermal interchange of ions. While it is possible that certain ions may be able to diffuse throughout the lattice of certain crystals, in this instance, where the lattice is dense and the "diffusing" ion, phosphate, is large and multicharged, it seems more probable that ion migration is mediated through the presence of

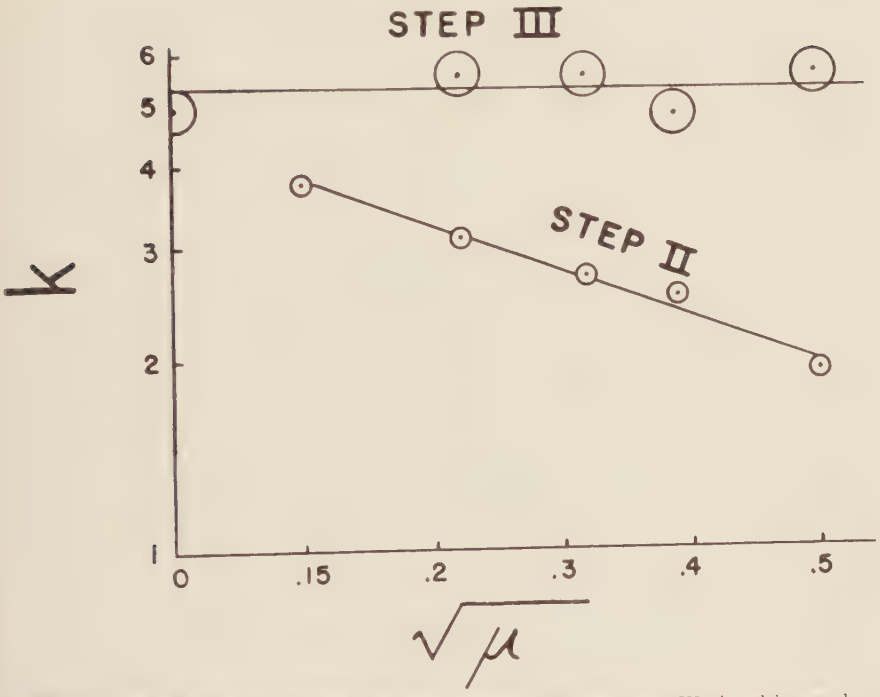

FIGURE 4. The effect of ionic strength on the rate constants for Steps II and III plotted in accordance with the Brønsted equation.

TABLE 1
PHYSICOCHEMICAL DATA ON THE EXCHANGE PROCESS

Variable	Effect on rate constant	
	Step I	Step II
μ	Varies inversely	No effect
$[PO_4^{=}]$	No effect	No effect
$[P^{32}O_4^{=}]$	No effect	No effect
+ Charge on crystal	Varies inversely	—
E_a	4 kcal./mole	= or < 4 kcal./mole
ΔS^{++}	−7 e.u.	−9 e.u.

unoccupied lattice positions or "faults" in the crystals. This view is supported by the fact that high-temperature ashing abolishes the recrystallization process.[2] Such heat-treatment renders the crystals more perfect, with fewer faults, as attested by improved X-ray diffraction patterns.[13]

The Determination of the Exchangeable Pool In Vivo

Whatever the mechanism by which bone mineral recrystallizes, the biologist is most interested in the quantitative significance of this process. How much

bone mineral recrystallizes *in vivo*? Unfortunately, the problem of evaluating *in vivo* the magnitude of the three processes of isotope-fixation, namely: exchange, recrystallization, and growth, has never been successfully resolved. When only a single injection of radiocalcium or radiophosphate is administered, the situation has proved almost hopelessly complex. All three variables are interacting. The SA of blood falls precipitously during the early time periods and, if one waits until the SA of blood has stabilized somewhat, redistribution of previously deposited isotope is a complicating factor.

The only available quantitative information on the recrystallization process has been derived from radioisotopic studies *in vitro*. These studies have shown a marked dependence on the type of material used. Some fresh bone preparations -selected areas of new bone growth—recrystallize at an amazing rate and to an amazing extent—almost completely in just a few days.[2] On the other hand, the recrystallization of aged, stable crystals is relatively slow and does not involve much of the total mineral. For example, the results of a long-term experiment using Ca^{45} and a commercial hydroxyapatite[8] is given in FIGURE 5. After 44 days, the total amount of calcium from the crystal which

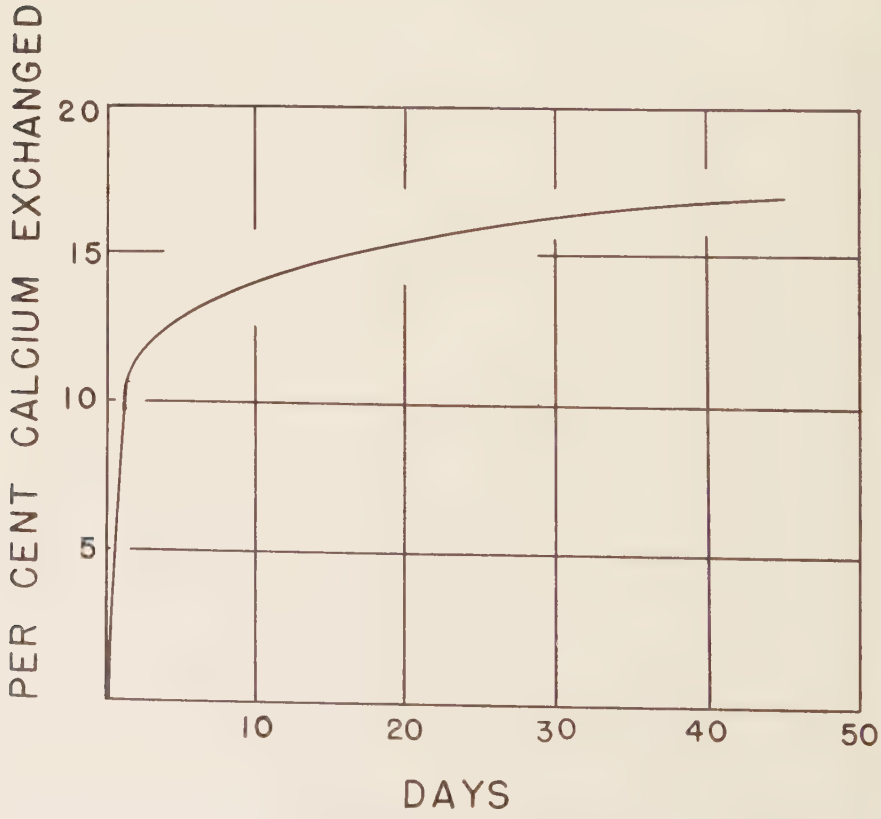

FIGURE 5. A long term exchange using Ca^{45}. Note that the transfer of isotope from solution to crystal occurs with ever-decreasing rate.

took part in the equilibrium was slightly less than twice the amount exchanged in the first day.

These results point up the need for definitive animal studies. At the moment, it is possible to generalize only in the most approximate terms. In the adult animal, nearly all of the crystals of bone have been aged for considerable periods of time. In the adult, we may expect, therefore, that "surface exchange" and "recrystallization" are about equal in magnitude (FIGURE 5), involving only a few per cent[13] of the total skeletal mineral. In the young, growing animal, however, with a large proportion of the skeleton made up of newly formed crystals, recrystallization must be of considerable quantitative importance. One can only guess at the percentage of skeletal mineral involved. Even surface exchange is greater in the young animal because of the greater hydration and better circulation in the young skeleton.[13]

Some of the difficulties of whole animal experimentation can be avoided by the administration of a diet of constant SA for an extended period. This technique was first reported by Kon and associates,[16] who studied the isotope distribution in various bones by means of radioautography. With this innovation, the SA of the blood, the extracellular fluid, the exchangeable fraction bone, the new calcification, and the calcification in reforming Haversian systems are all equal after an initial equilibration period of a few days. The problems of interpretation are greatly simplified, as a consideration of FIGURE 6 will show.

Haversian remodeling of *pre-existing* osteones will result in the resorption on nonradioactive crystals and the deposition of new crystals having an SA equal to that of the blood. If, under the experimental conditions, the rate of Haversian replacement remains constant, a linear increase in the SA of the skeleton with time will result, as shown in FIGURE 6A.

Ionic exchange and recrystallization, though continuing throughout the experiment, will cease to contribute to the increasing radioactivity of the skeleton, once the readily exchangeable pool has come into equilibrium. We are not troubled here by the fact that newly-formed crystals recrystallize at a rapid and continuing rate. Such newly formed crystals will be completely radioactive throughout (SA = Blood SA) and their recrystallization will not contribute additional activity. The SA of the skeleton resulting from exchange and recrystallization will rise sharply with time and will approach a limiting value asymptotically. In FIGURE 6A, the curve is drawn parallel to the abscissa for sake of simplicity.

New growth will result in the formation of crystals having an SA equal to that of the blood. If the rate of accretion of new mineral is constant throughout the experiment, the SA of the skeleton will increase linearly with time as indicated in FIGURE 6B.

The over-all relationship (FIGURE 6C) between radioactivity and time is the combination of all three rate processes which can be evaluated separately. By an extrapolation to zero time, the percentage of skeletal calcium undergoing surface exchange and recrystallization (the exchangeable pool) is given by the intercept $\left(\frac{\text{SA intercept}}{\text{SA blood}} \times 100\right)$. Parallel to the observed curve, a line which passes through the origin represents the SA changes resulting from remodeling

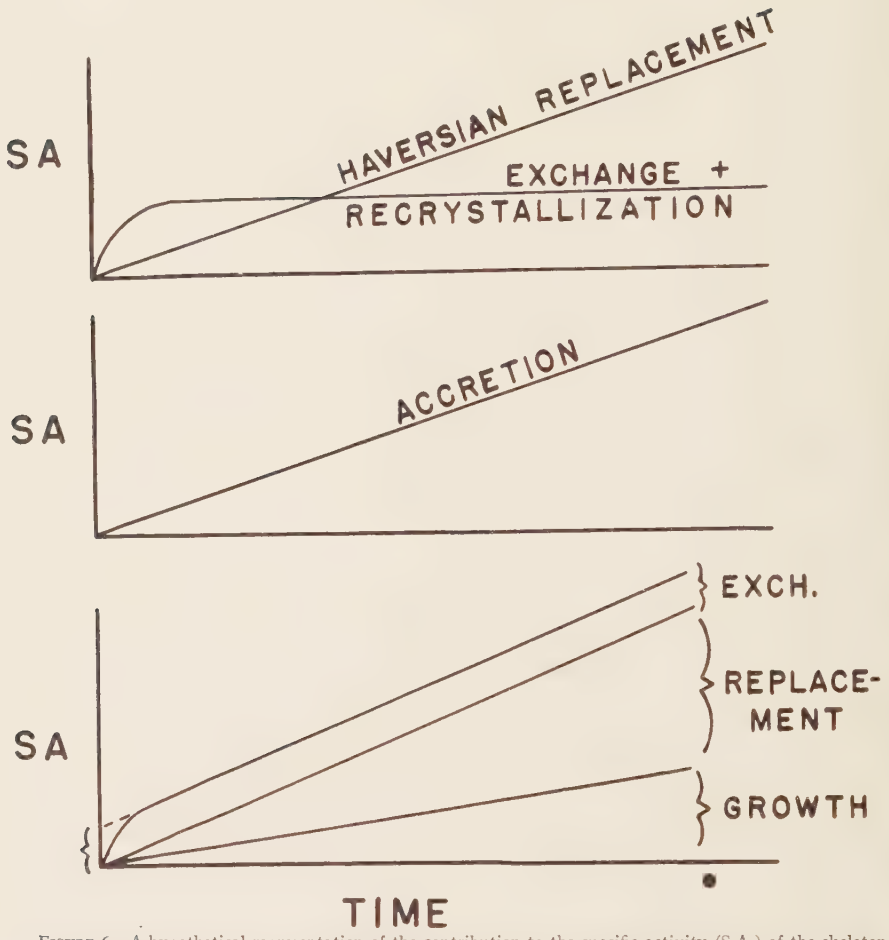

FIGURE 6. A hypothetical representation of the contribution to the specific activity (S.A.) of the skeleton of the three processes: exchange (6A), remodeling (6B), and net growth (6C), when animals are placed on a diet containing a constant level of radiocalcium. Note that the pool of exchangeable calcium may be derived from an extrapolation of the combined curve to zero-time.

and growth. The contribution of net growth can be evaluated from separate determinations of the actual increases in ash weight of the skeleton.

The experiments which test these theoretical relationships are just now in progress. A preliminary study on rats weighing 200 gm. (adult, but growing) has been completed, however, and the results, graphically presented in FIGURE 7, support the theoretical development just given. While too few anmals were used (four per experimental point) to permit an accurate evaluation, much of the radiocalcium accumulated by the skeleton resulted from accretion of new mineral over the 70-day period. Haversian replacement accounted for only 2.5 per cent in 70 days. The exchangeable pool is of the order of 4 per cent of the total skeleton as estimated from femur. While this is lower than some of the values postulated in recent years, it is entirely reasonable. For example, the new suggestion[13, 17] that the mineral crystals of bone form on an

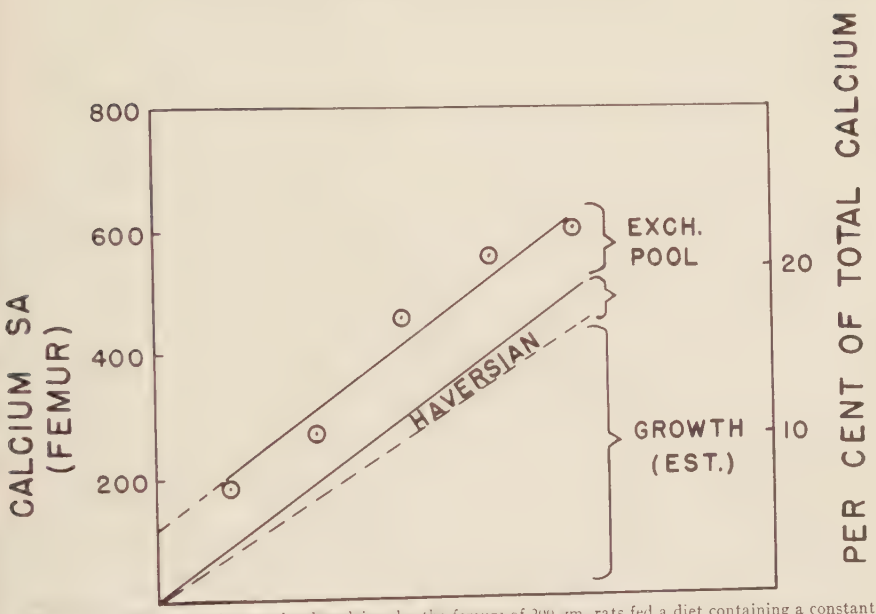

FIGURE 7. The accumulation of radiocalcium by the femurs of 200 gm. rats fed a diet containing a constant level of Ca^{45}.

organic template and recent electron microscopic evidence that the crystals lie *flat* on the collagen fibers,[18] means that only half the crystal surfaces could be exposed to fluid interchange. Also, recent evidence that compact bone is not fully hydrated[14] and that not more than a third of the skeleton is "available"[13] imposes a further reduction on the active surface area. Multiplying these two factors by the percentage (15 per cent) of surface mineral estimated from crystal size[18] one obtains: $\frac{1}{2} \times \frac{1}{3} \times 15$ per cent = 2.5 per cent available surface. Since, as discussed above, only aged crystals are contributing to the exchangeable pool *as measured*, FIGURE 5 suggests that an amount about equal to that surface-held undergoes recrystallization. Doubling the surface value (2.5 per cent) to include the recrystallizing fraction, one obtains 5 per cent as the predicted total exchangeable pool, in excellent, perhaps fortuitous agreement with the value obtained directly *in vivo*.

One interesting piece of information that can be derived from these data concerns the state of the CO_2 in bone. This important problem is clarified by a comparison of the results of the present study with the published report of Buchanan and Nakao.[19] Their animals were similar; their technique analogous. In place of the diet of constant SA, they exposed rats to an atmosphere of $C^{14}O_2$ of constant SA.

If their data are recalculated in terms of the theoretical considerations just given, one obtains the curve presented in FIGURE 8. Extrapolation of their observed data to zero time shows that over 30 per cent of the skeletal CO_2 is in the exchangeable pool! This suggests that all of the CO_2 in the "available" skeleton ($\frac{1}{3}$) is situated in the exposed surfaces of the crystals or in the layers immediately beneath. While some uncertainties remain concerning details,

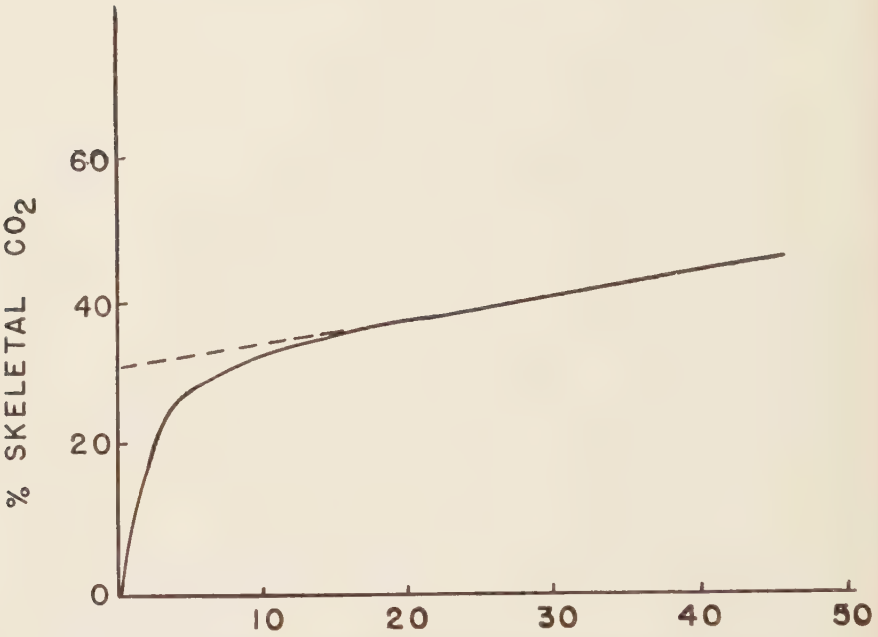

FIGURE 8. A plot of published data (Buchanan and Nakao[19]) on the accumulation of labeled CO_2 in the skeletons of mature rats breathing air containing CO_2 of constant specific activity.

this is a real advance in the long struggle[13] to determine the nature and site of the CO_2 in bone.

An Evaluation of the Dynamics of Calcium Metabolism

It has been shown how the over-all metabolism of calcium can be explained in terms of three general processes: growth, remodeling, and exchange. The discussion has emphasized the dynamic aspects. To appreciate fully the rapidity with which the circulating calcium "turns over" it is necessary to consider quantitative relations.

Assuming for the rat that:

Body weight = 100 gm.
Extracellular compartment = 25 per cent body weight
Extracellular compartment = 0.005 per cent Ca
Skeleton = 10 per cent body weight
Skeleton = 20 per cent calcium
Maximum growth rate = 10 per cent/day
Exchangeable skeletal calcium = 4 per cent
Haversian replacement = 0.03 per cent
Food intake = 10 gm./day
Food intake = 2 per cent Ca

and assuming that relative proportions *are not altered with age*, then the quantitative relations for calcium are given as follows:

(a) Blood	0.3	mgm.

(b) Haversian replacement	0.6	mgm./day
(c) Extracellular compartment	1.25	mgm.
(d) Exchangeable pool in bone	80	mgm.
(e) New bone formation	0 to 200	mgm./day
(f) Dietary intake	200	mgm./day
(g) Skeleton	2000	mgm.

In the young animal, the growth process dominates the dynamics of calcium metabolism in the skeleton. In a 24-hour period, the net accumulation of calcium (e) in new crystals alone exceeds in amount that in the exchangeable pool (d) and in the body fluids (a + c) by factors of 2 and 140 respectively. It must be stressed that this net accumulation neglects all the resorption, translocation, and redeposition mechanisms which are involved in the actual growth process. Also the newly deposited crystals recrystallize at a remarkable rate and are not included in the exchangeable pool (d) as here defined.

In adult, nongrowing animals (rats, unfortunately, rarely fail to grow), the pattern observed is dependent on the duration of the observation. In short-term experiments, the exchangeable pool (d) is dominant. With growth (e) negligible, the exchangeable pool is, in quantity, 50 times that in the body fluids (a + c). Dilution by dietary calcium (f) becomes increasingly important with passing time and, over long periods, Haversian replacement (b) assumes quantitative significance.

It is clear that much remains to be learned concerning the blood bone equilibrium. It is clear also that critical experiments involving radioisotopes and intact animals are exceedingly difficult to design and to conduct. Nevertheless, considerable clarification of our understanding of bone metabolism has taken place in recent years. The gross aspects of calcium metabolism in the skeleton can now be explained rationally in terms of three processes, namely: surface exchange, recrystallization, and growth (with attendant remodeling). Perhaps, as knowledge improves and as experimental techniques become more refined, current hypotheses will require modification. At least, these hypotheses are now sufficiently defined to be tested experimentally.

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X-RAY DIFFRACTION OF BIOLOGICAL AND SYNTHETIC APATITES*

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The improvement during the last 5 to 10 years in the design of X-ray diffraction equipment gives us an opportunity to re-examine our concepts of the structure of calcified tissues and the structure of the apatites. The microbeam techniques now permit the study of the crystal species and crystal orientation in tissue sections on areas as small as 30 microns.[1] Further reduction of the area is possible, but entails considerable inconveniences. When accompanied by a corresponding reduction in the thickness of the section, then the amount of diffracting matter which is irradiated by the beam is so small that the exposure times are prolonged to several hundred hours. The microfocus X-ray diffraction tubes of high brilliance can reduce the exposure times by a factor of 50. The new Geiger counter X-ray diffractometer, with its higher precision in the measurement of the reflection angles and the higher sensitivity in the detection of weak reflections, is essential in the study of the chemical composition of the apatites.

The only crystal species found in the mineralized tissues of vertebrates, *i.e.*, in enamel, dentin, cementum, and bone, is an apatite. In spite of pressing the search to the finest details, no reflection has been found on the diffractograms indicating the presence of another crystalline compound. Naturally, for the X-ray diffraction studies, dental enamel has been chiefly used, since its apatite is much better crystallized than the apatite of bone.

The crystallites in the tissues are oriented, forming "fiber" structures. In human incisor enamel, usually two fiber structures are observed intersecting at angles up to 60°. In shark's enamel they intersect at right angles.[2] The physical properties of the enamels are conditioned by this orientation. The factors directing the orientation must be found in the organic matrix. The diffractograms of dentin, cementum, and bone usually do not reveal such an orientation, as the individual protein fibers of the matrix are oriented in many directions. However, if special small areas are selected in which histological methods have shown a parallel orientation of the protein fibers, then X-ray examination will also reveal the preferred orientation of the apatite. In certain special varieties of cementum and bone, a high degree of parallel orientation of the apatite crystallites has been observed.

The size of the crystallites is estimated from broadening of the diffraction lines. Thus, in human incisor enamel and in bone, one finds an average size of 600 and 200 Å.,[3] respectively, or of 870 and 290 Å.[4] The difference in the results obtained by the various investigators is chiefly due to differences in the

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reduction formulae for obtaining the single-line width from the width of the $K\alpha_1$-$K\alpha_2$ doublet line. Furthermore, the calculations of the size are based on the assumption that limitation of the crystallite size is the only cause of the line broadening. If, also, the distortion of the crystallites is considered as an additional line-broadening factor, then the size may be found to be still greater.

The crystallite size is the best criterion for the differentiation between enamel and dentin tissues.[5] In its early stages of formation, before it is fully calcified, enamel shows the sharp and oriented reflections of the typical enamel pattern. Shark's enamel, in spite of its supposed mesodermal origin, gives a typical enamel pattern. The size determines the surface area for the ion exchange reactions. The large surface area of the small crystallites enables the bone to function as metabolic reservoir for calcium and magnesium and phosphate, while the much smaller surface area and the better quality of the crystallites in the enamel is a favorable factor in its resistance to chemical attack.

Low-angle X-ray scattering is used for the study of the smaller form-elements in these tissues and of the orientation of large organic molecules (*e.g.*, collagen). Two attempts at determining the size of assumed smaller apatite particles were made[6, 7] but their interpretation is still very uncertain. This technique does not differentiate between a dispersion of strongly scattering particles in a lightly scattering medium and a dispersion of lightly scattering particles (voids or fibers) in a strongly scattering medium. A dispersion of calcite particles in apatite would give only a weak effect, as the electron density of calcite is only 15 per cent lower than that of the apatite. If the two phases have the same scattering power, then there will be no low-angle scattering effect.

The nature, composition, and structure of the biological apatites is one of our major concerns. The apatites are one of the crystallized forms of calcium phosphate. The atomic structure of the apatite was elaborated more than 20 years ago. The hexagonal unit cell, which is the smallest space unit of the structure containing all the crystallographic symmetry elements of the whole crystal, is a parallelopipedon whose edges are formed by the two horizontal a axes, enclosing an angle of 120°, and by the vertical c axis at right angles to the a axes. This unit cell contains 10 Ca^{++}, 6 $PO_4^{\equiv}$, and 2 OH^- ions. The phosphate oxygens are arranged in tetrahedral groups enclosing the phosphorus and are tied more strongly to it than to the Ca^{++} ions which are interspersed between the $PO_4^{\equiv}$ groups holding them in the structure together. When the crystal grows from the solution, the $PO_4^{\equiv}$ groups as such are built into the crystal. The two OH^- ions sit on the hexagonal c axis, each surrounded by three Ca^{++} ions at the same level. The other four Ca^{++} ions occupy positions on the two vertical trigonal axes, which pass through the cell at one third and two thirds along the long cell diagonal.

When an ion in this structure is substituted by another ion of different size, then the unit cell dimensions are affected by this substitution; *e.g.* replacement of OH^- by F^- slightly shortens the a axis (.013 Å. per one weight per cent F), replacement by Cl^- lengthens the a axis and slightly shortens the c axis. It is our working hypothesis that changes in the unit cell dimensions are caused by

substitution. On this basis, the X-ray determination of the cell dimensions (*i.e.*, length of *a* and *c* axes) can provide clues with regard to the chemical composition of the apatite structure proper, without the interference of material adsorbed to the surface of the crystallites. Chemical analysis cannot make this distinction.

According to their origin, we may divide the apatites into four groups: (1) mineral apatites; (2) synthetic apatites formed or treated at elevated temperatures; (3) synthetic apatites precipitated from aqueous solutions; and (4) biological apatites. As biochemists, we are interested particularly in the biological apatites; but from the experimental work with each of the other groups, certain essential features of the apatites have been derived which could not have been recognized if we had worked with material from one group alone.

(1) *Mineral apatites.* Among the naturally occurring apatite minerals, we find some which are beautifully crystallized. Well crystallized materials are best suited for X-ray work, as more precise information can be obtained from them. The atomic structure of the apatite has been determined from X-ray data obtained with such mineral apatites. Sometimes they contain appreciable amounts of carbonate; then they are carbonate apatites. Francolite is the name for the carbonate fluorapatites, and dahllite for the carbonate hydroxy apatite. We are much interested in these carbonate apatites, since the biological apatites also contain carbonate.

The problem of placing the carbonate within or without the apatite structure is an old problem, and it is still debated. The adsorption of calcium carbonate on the surface of the apatite crystallites in bone and phosphate rock is propounded by Hendricks.[8, 9] On the other hand, McConnell, working with minerals, came to the conclusion that the carbonate is a substituent within the apatite structure, substituting for Ca and PO_4 groups. According to his latest concept,[10] four flat $CO_3^=$ groups replace three tetrahedral $PO_4^{\equiv}$ groups around the trigonal axis in such a way that one CO_3 group sits on the axis, parallel to the basal plane, while the other three CO_3 groups stand vertically around the axis. Thus, the positions of the oxygens in both structures, apatite and carbonate apatite, are about the same. The nearest Ca^{++} on the axis is replaced perhaps by water or leaves a vacant site. The one negative charge which now remains in excess can be disposed of in various ways to retain electrostatic neutrality.

Though some of these apatites contain 6 weight per cent (and more) CO_2, corresponding to 13 weight per cent $CaCO_3$, no inclusions are visible with the microscope. Even with X-ray diffraction, no calcium carbonate reflections can be recognized, while, in a mechanical mixture, 1 per cent of well crystallized calcite can be definitely detected with X rays. This finding speaks for the substitution concept. However, in defense of the adsorption and occlusion concept, it may be said that, if the carbonate is poorly crystallized, *i.e.*, if it forms distorted and small crystals, say 200 Å. in size, then the peaks of the reflections are lowered and broadened and the detectable limit may easily be raised to 10 or 20 per cent.

The lower density and the lower average refractive index (and the other

optical properties) of the carbonate apatites as compared to the carbonate free apatites cannot be quoted as support for the substitution concept, as the occlusion of submicroscopic calcite particles would produce the same effects.

The low negative birefringence of the apatite is increased in the carbonate apatite. This increase is attributed to the orientation of the flat carbonate groups parallel to the basal plane of the apatite. We may calculate how many CO_3 groups must be thus oriented in order to produce the observed birefringence. From a compilation of Geiger[11] we take, as an average for the various observations, an increase in the birefringence of 0.0072 per 4.4 per cent CO_2 (= 10 per cent $CaCO_3$) content. In calcite, the CO_3 groups are all oriented parallel to the basal plane. Its birefringence, therefore, is high, namely 0.172. An increase in the apatite's birefringence of 0.0072 could then be produced by the presence of 4.2 per cent of oriented calcite. This possibility means that approximately 40 per cent of the available CO_3 groups in the carbonate apatite must be oriented parallel to the basal plane in order to produce the observed increase in the birefringence. The remaining 60 per cent of the CO_3 groups must have random orientation. If we take McConnell's substitution picture literally, then only 25 per cent of the CO_3 groups are parallel to the basal plane, which gives too low a birefringence; this is lowered still further and changed into a positive birefringence by the 75 per cent CO_3 groups which are parallel to the vertical axis. Here McConnell's picture of substitution does not agree so well. Thus, the birefringence can give us a clue to the orientation of the CO_3 groups, but does not tell whether they substitute in the apatite structure or whether they are contained in submicroscopic $CaCO_3$ particles. Neither, we think, can this issue be decided definitely on the basis of other optical properties, as, for instance, the biaxial behavior, which is observed in some carbonate apatite crystals.

Fluor apatite has its principal cleavage parallel to the basal plane. The carbonate apatites are often fibrous parallel to the *c* axis, or they exhibit a pronounced prismatic cleavage. If the carbonate is preferentially adsorbed to the prismatic planes of the growing apatite crystal, interfering with the further deposition of calcium phosphate on these surfaces, then we can expect a change in the habit from tabular or chunky towards fibrous.[13] Here too, however, the predominant orientation of the flat CO_3 groups parallel to the *c* axis would produce a positive instead of a negative birefringence.

The most important support for McConnell's concept of CO_3 substitution in the apatite structure comes from X-ray diffraction studies, but not because he found the intensities in the X-ray diffraction patterns of francolite different from those of F apatite.[10] We have also observed these intensity differences and have identified them as being caused by a packing orientation of the unequilateral powder grains in the samples placed into the diffractometer and, in turn, as due to the above-mentioned different cleavage behavior of the two apatites. But it is important that McConnell found the unit cell dimensions of the carbonate apatites smaller than those of the carbonate free apatites.[14] He found the *a* axis in francolite 0.02 kX shorter than in F apatite and, in dahllite, 0.01 kX shorter than in OH apatite.

TABLE 1

APATITE UNIT CELL DIMENSIONS

No.	Specimen	F wt. %	CO_2 wt. %	*a* axis Å.	*c* axis Å.
1	Francolite, Staffel**	4.11	3.36	9.340	6.890
2	Francolite, Cornwall**			9.354	6.884
3	Francolite, Devonshire**			9.356	6.881
4	F apatite, Ontario*	3.40	.45	9.375	6.890
5	F apatite, Durango*	3.27	.24	9.387	6.880
6	Dahllite, Wyoming***			9.394	6.890
7	Dahllite, Wyoming***			9.391	6.898
8	F apatite, synth., standard*	3.77	0	9.373	6.882
9	OH apatite, synth., standard*	0	0	9.421	6.881
10	OH apatite, carbonated**	0	1.3	9.448	6.881
11	Dental enamel, human***	.01	2–3	9.441	6.884

IDENTIFICATION OF SPECIMENS ✱

No. 1. Francolite, Staffel, Germany. U. S. Nat. Mus. No. 97339.
Very light olive translucent fibrous crust, 5 mm. thick. Analysis in McConnell.[14] On brown opaque rock. The diffraction pattern of this rock shows that it consists of apatite and quartz. This apatite is less well crystallized, but has the same axes as the fibrous crust, *i.e.*, it is also francolite.

No. 2. Francolite, Cornwall, England. U. S. Nat. Mus. No. 97338.
Colorless clear prisms. 1.5 mm. long, 1 mm. thick, on quartz.

No. 3. Francolite, Devonshire, England. Harvard No. H89618.
Colorless clear prisms, 1.5 mm. long, 1 mm. thick, on quartz.

No. 4. F apatite, Turner Mtn. in Lake Clear, near Eganville, Renfrew Co. Ontario.
More or less clear, greenish, small crystals picked out from among large redbrown apatite crystals in pink calcite. Analysis No. 1 in Dadson.[15] The X-ray pattern of the large red-brown crystals reveals the same apatite axes and two weak quartz lines.

No. 5. F apatite, Durango, Mexico.
Large clear yellow-green crystals. Analysis by R. C. Likins, National Institute of Health, for material U. S. Nat. Mus. No. 104021; Ca = 40.5%, P = 18.6%.

No. 6. Dahllite, Ishawooa, Wyoming. U. S. Nat. Mus. No. 103219.
Brown fibrous spherulitic concretions. Description in McConnell.[16]

No. 7. Dahllite, Bighorn Basin, Wyoming. U. S. Nat. Mus. No. 104109.
Lightbrown fibrous spherulitic concretions.

No. 11. Mixed sample from various teeth.

Axis values calculated from 410 and 004 reflections. CuK α radiation, Norelco Geiger Counter X-ray Diffractometer, calibrated with silicon standard. The angular positions of the reflections on the various charts were compared with those of the standard OH and F apatites by superimposition of the charts.

* Very well crystallized materials; α_1-α_2 doublet resolved. Maximum deviation ±0.003 Å. Here, the precision is limited chiefly by the surface quality (flatness) of the specimens placed in the diffractometer. Extreme care was used in their preparation.

** Well crystallized materials; α_1-α_2 doublet not resolved. Maximum deviation ±0.005 Å.

*** Fairly well crystallized materials. Maximum deviation ±0.006 Å.

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The new X-ray diffractometer is able to give us a higher precision than was obtainable 15 years ago. We have measured with it the axes of various apatite materials and have compiled the values in TABLE 1, together with the F and CO_2 contents, as far as they are known. These values show even a greater shrinkage of the apatite, when carbonate enters it. In francolite (Nos. 1, 2, 3, TABLE 1) the *a* axes are 0.035 and 0.02 Å. shorter than in F apatite (Nos. 4, 8); and, in dahllite (Nos. 6, 7), they are 0.025 shorter than in OH apatite (No. 9). As stated above, it is our working hypothesis that we would consider any changes in the cell dimensions as an indication of substitution. The changes

will be approximately proportional to the concentration of the substituent as long as it is randomly distributed among the available positions. Thus, by comparison of the francolite from Staffel (No. 1) and the synthetic F apatite (No. 8) and OH apatite (No. 9), we calculate an approximate shrinkage of the a axis by 0.013 Å. for 1 per cent F, and by 0.0086 Å. for 1 per cent CO_2 contained in the francolite (or by 0.010 Å. for 1 per cent CO_2, when the 0.34 per cent F above the theoretical content in F apatite does not cause any further shrinkage).

Since we do not have any other plausible explanation for the shrinkage, we shall consider the carbonate apatite as a product of substitution. A shrinkage seems plausible, if one compares the size of the plane CO_3 group with the size of the triangular base of the PO_4 tetrahedron, for which the CO_3 is supposed to substitute. The oxygen-oxygen distance in a CO_3 group is 15 per cent smaller than the oxygen-oxygen distance in a regular PO_4 group.

When the carbonate apatites are ignited, they lose CO_2, and their a axes change in the direction of the CO_2 free F and OH apatites.

(2) *Synthetic apatites, made at elevated temperature.* Since the mineral apatites usually contain various impurities, it was necessary to synthesize a supply of pure materials so that we could study the properties of the pure apatites. Precipitates from aqueous solutions had variable compositions, were often poorly crystallized, and had variable unit cell dimensions. Ignition (in a muffle furnace for 16 hours at 900° C.) of a finely powdered mixture of β-tricalcium phosphate and calcium hydroxide gave an apatite whose crystallinity was good but which contained considerable amounts of the unreacted educts, interfering with the measurement of the axes.

We also heated the same mixture in an autoclave at 360° C. for 16 hours with steam of 200 atm. pressure. This amount of pressure is little below the critical point of water. A platinum crucible with the reaction mixture was suspended in the upper part of the autoclave chamber. The steam prevents the premature loss of water from the hydroxide. The reaction scheme is shown in TABLE 2. As starting material, we used whitlockite (β-tricalcium phosphate) which we had previously prepared by ignition at 900° C. of commercial (Baker's C.P. analyzed) so-called tertiary calcium phosphate (Ca/P = 1.50). The product was fairly well crystallized apatite. On ignition, a small amount of whitlockite reappeared which diminished by repeated autoclaving and ignition. Thereby the crystallinity of the material was improved, becoming very good, as can be judged from the resolution of the α_1-α_2 doublet of the 410 and 004 reflections on the diffractometer traces. The cell dimensions of

TABLE 2

PREPARATION OF SYNTHETIC APATITES IN THE AUTOCLAVE

(1)	β-$Ca_9(PO_4)_6$ + $Ca(OH)_2$	$\rightarrow$ $Ca_{10}(PO_4)_6(OH)_2$
(2)	β-$Ca_9(PO_4)_6$ + CaF_2	$\rightarrow$ $Ca_{10}(PO_4)_6F_2$
(3)	β-$Ca_9(PO_4)_6$ + $CaCl_2$ + nH_2O	$\rightarrow$ $Ca_{10}(PO_4)_6(Cl, OH)_2$ + $nHCl\uparrow$
(4)	β-$Ca_9(PO_4)_6$ + $CaCO_3$ + H_2O	$\rightarrow$ $Ca_{10}(PO_4)_6(OH)_2$ + $CO_2\uparrow$
(5)	β-$Ca_9(PO_4)_6$ + $CaCO_3$ + nH_2O	$\rightarrow$ "Carbonated" OH apatite
	CO_2 pressure	

the first autoclave product are not changed by ignition, or by repetition of the autoclave and furnace treatment, as is ascertained by superimposition of the diffractometer curves. Also, after five repetitions of the furnace treatment, the axes were still the same, and we find the same axes in the above-mentioned product of the direct ignition of whitlockite and calcium hydroxide, without previous synthesis in the autoclave. Furthermore, when an excess of whitlockite or of calcium hydroxide is used in the preparation, the apatite axes are also found to have the same length. Here we have a method of preparing an OH apatite which we can use as a standard in our X-ray diffraction work. The axes of this apatite have been listed in TABLE 1, No. 9.

The standard F apatite (No. 8) was prepared and checked by the same methods (TABLE 2, EQUATION 2). Excess of whitlockite or of calcium fluoride in the reaction mixture did not influence the axes. Cl apatite can not be obtained pure in the same way, as some of the chloride is hydrolyzed off by steam (EQUATION 3). Probably a mixed (Cl, OH) apatite results. Also, when one tries to make a carbonated apatite by the same method, the CO_2 is lost and the product has the axes of the standard OH apatite (EQUATION 4). When the CO_2 loss was counteracted by a CO_2 atmosphere in the autoclave (EQUATION 5) we obtained an apatite with a different *a* axis. It is 0.02 Å. longer than that of the OH apatite (TABLE 1, No. 9). The material contained 1.3 per cent CO_2 (= 2.9 per cent $CaCO_3$). We cannot yet say why the CO_2 causes here an expansion of the unit cell, while in the mineral carbonate apatite it causes a contraction. Perhaps some water enters the structure with the CO_2. We have repeated the experiment and observed the same expansion. No carbonate line could be detected with X rays. Ignition of this material or treatment in the autoclave (without the CO_2 atmosphere) causes the loss of CO_2 and a return of the cell dimensions to those of the standard OH apatite. It is of particular interest that the cell dimensions of this "carbonated OH apatite," as we may temporarily call it, are almost the same as those of the apatite in human dental enamel.

(3) *Synthetic apatites precipitated from aqueous solutions.* Since the biological apatites are formed by precipitation of calcium phosphate from aqueous solutions, we started our investigation of the substitution effects with the preparation of a great number of calcium phosphate precipitates made under various conditions of acidity and temperature and concentrations. All these precipitates were examined in the diffractometer in their original state (air dried) and after treatment in the autoclave and after ignition. All peaks on the diffractometer curves have been definitely identified. The diagram of FIGURE 1 shows the various forms of calcium phosphates which have been observed under these conditions, together with their Ca/P ratio on a molar scale. The weight ratio of Ca/P is 1.29 times the molar ratio. We have obtained among the precipitates some whose diffraction patterns were that of an apatite alone, though their Ca/P ratio deviated considerably from 1.67, as far as 1.33 and 2.0 or more. There were, of course, also many precipitates with a Ca/P ratio below 1.67 whose diffraction patterns showed the presence of the secondary phosphates brushite or monetite besides the apatite. Some precipitates

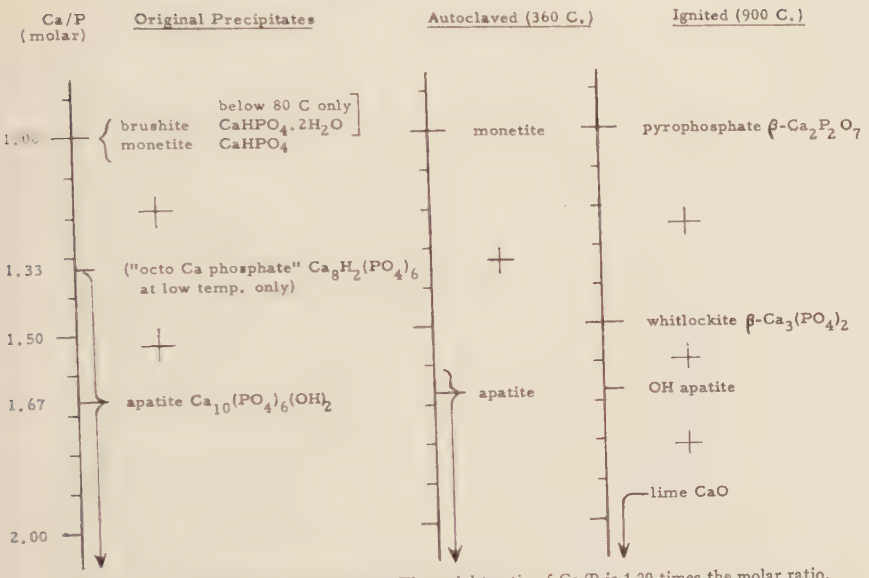

FIGURE 1. Forms of calcium phosphates. The weight ratio of Ca/P is 1.29 times the molar ratio.

contained an intermediary phase with a characteristic diffraction pattern of its own. The most outstanding reflection of this phase comes from planes with a spacing of 18.87 Å. which is double the length of the *a* axis of the precipitated OH apatite. Two other reflections of it (d = 3.412 Å. and d = 2.847 Å.) almost coincide with the 002 and the 211 reflection of the precipitated apatite (d = 3.44 Å. and d = 2.81 Å.). This phase is the same which Warington[17] and Arnold[18] described as "octocalcium phosphate." We hesitate, however, to apply this name since, although the Ca/P ratios of our precipitates were near 1.33, the diffraction patterns showed the presence of considerable amounts of poorly crystallized apatite. The presence of larger amounts of apatite is recognized also on the diffraction patterns which Arnold obtained from his "octocalcium phosphate" precipitates with a Ca/P ratio near 1.33. In the autoclave, the apatite precipitates whose Ca/P is below 1.67 split into monetite and apatite. Ignition of these precipitates produces β-pyrophosphate or whitlockite or OH apatite, or mixtures of two of these, depending solely on the gross Ca/P ratio of the material. If Ca/P is above 1.67, lime appears as second phase with the OH apatite.

From all these materials, we selected those whose diffraction patterns were of apatite, without another phase admixed, and plotted the cell dimensions against the Ca/P ratio of the precipitate (FIGURE 2). On the upper half of this graph, the length of the *a* axis is recorded; on the lower half, the length of the *c* axis. We see that, with decreasing Ca/P ratio, the *a* axis becomes longer, the *c* axis perhaps slightly shorter. The points are scattered more than the error limit of ascertaining the axis lengths justifies, showing that other factors besides the Ca/P ratio influence the axes. After the materials have been autoclaved or ignited their axes are often found somewhat shorter.

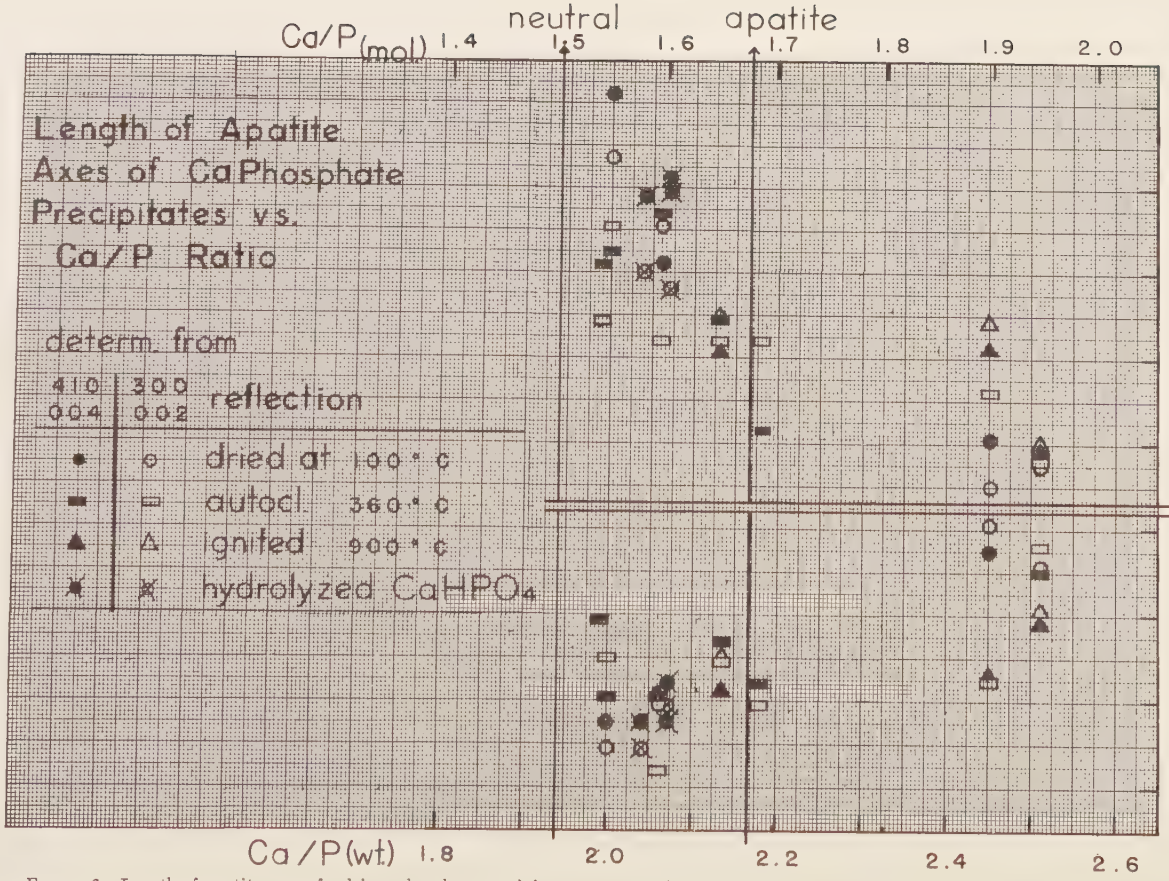

FIGURE 2. Length of apatite axes of calcium phosphate precipitates versus Ca/P ratio. The values for the a axes are plotted in the upper half, those for the c axes in the lower half of the graph.

The variations of the unit cell dimensions in these precipitated apatites convinced us that we hardly should find the "standard" OH apatite among the precipitates. We therefore interrupted this series and tried the preparation of the standard at elevated temperatures which has been described in the previous chapter. The precipitation experiments, as far as they have gone, are of an exploring nature, scanning a wide field of experimental variations. They are not the final proof of a preconceived hypothesis; but, at the present state of the investigation, we may try to develop a concept which explains the observations made so far and can guide us in the planning of future experiments with calcium phosphate precipitates.

The first problem may be formulated as follows: what substitution in the apatite causes the observed variations of its axes? It has been considered that isomorphous substitution of one Ca by hydrogen in OH apatite, $Ca_{10}(PO_4)_6(OH)_2$, would produce a tricalcium phosphate hydrate, $Ca_9H_2(PO_4)_6(OH)_2$ (inconveniently also named α-tricalcium phosphate).[19, 20] The Ca/P ratio is thereby lowered from 1.67 to 1.50. Substitution of two Ca would produce a material of the composition of octocalcium phosphate, $Ca_8H_4(PO_4)_6(OH)_2$, with a Ca/P ratio of 1.33. Such a substitution should be accompanied by a considerable change in the length of the axes, since the substitution of OH^- by F^- (two ions as like in size as two can be) produces a change of the a axis of 0.05 Å. We should also expect a contraction rather than the expansion which we observe. There are also other objections to this type of substitution: (1) the size of the H^+ ion is so much smaller and, therefore, the polarization force upon its neighbors so much greater that, in an inorganic structure, such a substitution of a metal ion by the H^+ ion never happens without the structure changing to a different type of lattice;[21] (2) the coordination of the H^+ ion in crystals is never more than twofold,[21] while that of Ca^{++} is sixfold and when the H^+ ion enters the crystal (perhaps with an $HPO_4^=$ ion), it would most probably take a position between two oxygens of adjacent PO_4 groups, pulling them somewhat closer together; (3) in OH apatite there are two different kinds of positions for the Ca atoms; namely, 6 equivalent Ca^I surrounding the two OH on the hexagonal axis, and 4 equivalent Ca^{II} sitting on the trigonal axis. The formula $Ca^I_6Ca^{II}_4(PO_4)_6(OH)_2$ indicates the two types of Ca^{++} positions. The 6 PO_4 groups are all equivalent. The writing of the apatite formula $Ca_9(PO_4)_6 \cdot Ca(OH)_2$ has no crystallographic basis. There is no reason evident why the isomorphous substitution for Ca should stop at exactly one Ca, unless the substitution is not random and results in a superstructure or an entirely different structure. But no such substitution has ever been found in the range between the "octocalcium phosphate" and the OH apatite. For all these reasons, we must definitely discard the idea of the tricalcium phosphate hydrate as a crystal species of its own and as the product of an isomorphous substitution of H for Ca in the OH apatite. But the possibility of a substitution of Ca by hydronium (H_3O^+) has not been eliminated. However, in this case, the precipitates must contain additional amounts of water.

Let us therefore consider what information a study of the water content of the precipitates may give us regarding their constitution. In TABLE 3, we

TABLE 3
WATER CONTENT OF VARIOUS NEUTRAL CALCIUM PHOSPHATES
Ca/P = 1.50

Substitution in OH apatite	
(1) $Ca_9H_2(PO_4)_6(OH)_2$	$\rightarrow Ca_9(PO_4)_6 + 2\ H_2O$
(2) $Ca_9(H_3O)(PO_4)_5(HPO_4)(OH)_2$	$\rightarrow Ca_9(PO_4)_6 + 3\ H_2O$
(3) $Ca_9(H_3O)_2(PO_4)_6(OH)_2$	$\rightarrow Ca_9(PO_4)_6 + 4\ H_2O$
Occlusion in OH apatite	
(4) $\frac{3}{4}\ Ca_{10}(PO_4)_6(OH)_2 + \frac{6}{4}\ CaHPO_4 \cdot 2H_2O$	$\rightarrow Ca_9(PO_4)_6 + 4.5\ H_2O$
(5) $\frac{3}{4}\ Ca_{10}(PO_4)_6(OH)_2 + \frac{6}{4}\ CaHPO_4$	$\rightarrow Ca_9(PO_4)_6 + 1.5\ H_2O$

compare precipitates, all of which have the same Ca/P ratio (*i.e.* 1.50) on the assumption that one calcium of the OH apatite has been substituted in some manner by hydrogen (EQUATION 1), one hydronium (EQUATION 2), two hydroniums (EQUATION 3); or we can assume that the precipitates are formed by coprecipitation of the secondary phosphates brushite (EQUATION 4), or monetite (EQUATION 5), with the OH apatite. In every case, the amount of water given off by the precipitate on ignition should be different. Also, when the water contents of various precipitates made under comparable conditions are plotted against the Ca/P ratios, the slope of the curve may tell by which one of the five processes the precipitates are formed (FIGURE 3). A systematic experimental study of the water content of such phosphate precipitates has not yet been made. It is still on our program.

As one of the possible explanations for the varying Ca/P ratio of the apatite precipitates, we mentioned in the previous paragraph the coprecipitation of secondary phosphate with the apatite. By coprecipitation we mean, in a broad sense, the inclusion in the apatite precipitate of material from the solution which does not form by itself a structure isomorphic with the apatite. Such inclusions are to be considered as a consequence of local kinetic happen-

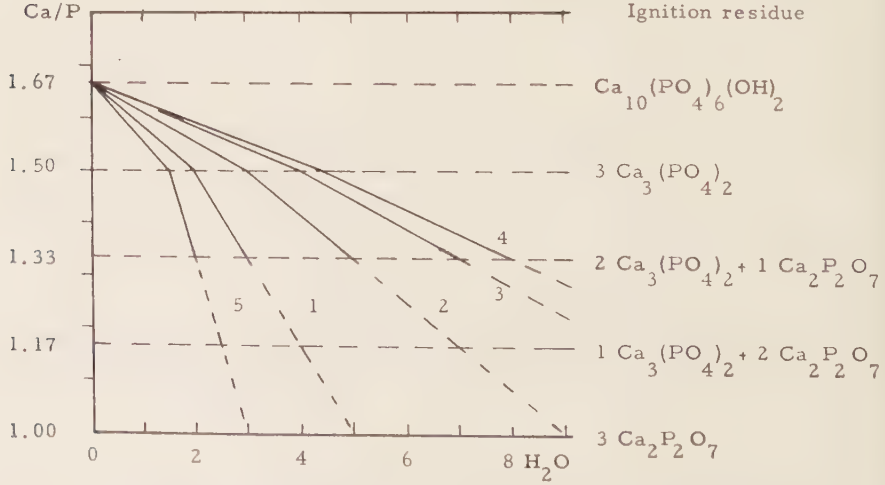

FIGURE 3. Expected water elimination on ignition versus Ca/P ratio of calcium phosphate precipitates. Curves 1 to 5 correspond to reactions 1 to 5 of TABLE 3 by which the variation of Ca/P is effected.

ings on the surface of the growing crystal and do not represent equilibrium conditions between the crystal and its surrounding solution. The experimental conditions of coprecipitation may vary within a wide range. On the one end of the range, we may consider a system in which a colloid (*e.g.*, a dye) suspended in the solution is occluded in the growing crystal, or where the precipitating agent forms a precipitate also with the cosolute (*e.g.*, the solution of a calcium salt added to a phosphate solution containing carbonate or sulfate). On the other end there is a system in which the concentration of the coprecipitate in the solution remains far below saturation, so that, by itself, it would not separate as a solid (*e.g.*, coprecipitation of sodium phosphate with the calcium phosphate).

It is readily understood that the speed of adding the precipitating agent (Ca salt) is one very important factor influencing the composition and the structure of the precipitate, particularly when (case 1) addition produces local supersaturation for both host (Ca phosphate) and coprecipitate (Ca carbonate), while (case 2) very slow addition may produce supersaturation for the host only, and the ion product of the coprecipitate remains below its solubility product. In the first case, increasing the carbonate/phosphate ratio in the solution eventually will change the role of the carbonate from coprecipitate to host, and the role of the phosphate from host to coprecipitate. Between these two conditions there is a range where we can expect a poorly crystallized or amorphous mixture of phosphate and carbonate. The greater the rate of addition of the precipitating agent, the wider is this range. In the second case, however, if at a low rate of calcium addition and at a low carbonate-phosphate concentration ratio, the solubility product of calcium carbonate is not reached, we cannot expect a nucleus of calcium carbonate to grow into a crystal, and still less can we expect a nucleus for such a crystal to form. Whenever, therefore, during the precipitation of the host, the ion product of the precipitating ions and the cosolute ions remains well enough below their solubility product, then we have to assume that any included coprecipitant is included in the host as individual ions rather than as particles.

Thus far we have discussed the probability of the coprecipitant's being dispersed as ions or aggregated into particles. Other features of the phenomenon of coprecipitation have been described by Walton and Walden,[22] who studied the coprecipitation of various univalent ions with barium sulfate. They precipitated barium sulfate between 92° and 98° C. by the slow addition of barium salicylate solution to neutral solutions of ammonium, potassium, sodium, or lithium sulfates, in varying concentrations. The precipitated barium sulfate contained up to 5 and 7 mole per cent of these univalent ions. Precision measurements of the unit cell dimensions of the barium sulfate revealed that a greater or lesser expansion, up to 0.2 linear per cent, accompanied the inclusion of these ions, depending on their relative radii. This inclusion, then, must be of the type of a substitution. When the univalent ions occupy the positions of the Ba^{++} ions, an equivalent number of $SO_4^{=}$ ions is replaced by HSO_4^{-} ions. Such a substitution may seem quite "normal." The investigators also found that, up to 20 mole per cent, water may be included, which causes a linear expansion of the $BaSO_4$ structure up to 0.15 per cent. This

expansion too, then, should be considered a substitution, however abnormal it may seem. The explanation made by the investigators is that a group of three water molecules functions as a substituting unit. At the density of liquid water, this group requires 4 per cent more space than the 1 $BaSO_4$, whose place in the structure it presumably occupies. On ignition above 500° C., this water is lost, but the structure remains expanded.

We are reporting here Walton and Walden's experiments with barium sulfate because they demonstrate what we should expect to happen also in the precipitation of the calcium phosphates. Furthermore, these experiments give us a possible explanation for an observation which we have made at many occasions; namely, that the precipitated apatites often have an axis which is 0.1 or 0.2 per cent (= .01 or .02 Å.) larger than that of the apatite made at high temperature. We like to explain this expansion, as in the case of barium sulfate, as being due to the coprecipitation of water. Since the precipitates from which the data in FIGURE 2 have been selected were made under a variety of conditions, their content of coprecipitated water also must have varied and have caused the scattering of the axis values. This amount of coprecipitated water we assume to be independent of that water which enters the structure as H_3O^+ or together with coprecipitated $HPO_4^=$, and we must consider also its influence when we study the substitution effects of various ions upon the unit cell dimensions of precipitated apatites.

The coprecipitation of a secondary phosphate near a neutral pH is readily anticipated, since the concentration ratio HPO_4/PO_4 is of the order of magnitude 100,000 to 1 and since the secondary calcium phosphates are only sparingly soluble. This coprecipitation, therefore, can explain how the Ca/P ratio of the apatite precipitate is lowered when Ca^{++} solution is added to an excess of phosphate solution. When phosphate is added to an excess of calcium hydroxide solution, some of the latter is coprecipitated and raises the Ca/P ratio above that of the apatite. The "adsorption on internal surfaces"[8,9] makes one visualize the coprecipitate concentrated more or less in certain layers of the host structure, tending there to break the coherence of the lattice and, thus, to limit its crystal size or to cause at least considerable distortion of the lattice planes. But the coprecipitate can also be dispersed more homogeneously throughout the host structure, even as individual ions. As in a normal substitution, these ions then assume an oriented position within the host structure and affect its unit cell dimensions. We anticipate that further coprecipitation experiments will bring about a reconciliation of the two originally antithetic concepts of "occlusion" and "substitution."

We may now reconsider the mineral carbonate apatites, regarding them as coprecipitates of carbonate with the phosphate. Some francolites, as we have noted, are well crystallized, give sharp X-ray reflections, though the α_1-α_2 doublet is not resolved. We may take this degree of crystalline perfection as indication that, during the growth of the crystals, the addition rates must have been very low. Under these conditions, the absence of calcium carbonate crystals indicates an insufficient ion product and, consequently, it also indicates that whatever carbonate is included, is included as individual ions. The orien-

tation of these ions is attested by the increased birefringence, and the shrinkage of the unit cell is evident.

The low distortion of the atomic planes in some of the mineral carbonate apatites was facilitated by the elevated temperature at which they were formed. On the other hand, due to the exceedingly low crystallization velocity of the apatite,[24] the precipitates from aqueous solutions at room temperature, or even at 100° C., are poorly crystallized unless the addition of the precipitating agent is exceedingly slow. If carbonate is present in the phosphate solution in increasing concentrations, the precipitated crystals are increasingly distorted, due to the simultaneous precipitation of the carbonate. Thus, from equimolar solutions, we have obtained precipitates whose X-ray diffraction pattern is just a straight line without any apatite or calcium carbonate peak. The material is completely amorphous, an unorganized heap of calcium and phosphate and carbonate ions.

(4) *Biological apatites.* The biological apatites are precipitates from aqueous solutions. These solutions contain, besides calcium and phosphate ions, also carbonate, chloride, citrate, magnesium, and sodium ions in appreciable concentrations. The presence of soluble and of insoluble proteins, and the presence of soluble unionized calcium and phosphate compounds with the appropriate enzyme systems for their hydrolysis is also of importance. Whatever we have said in the previous chapter about the calcium phosphate precipitates is applicable also to the biological apatites. However, on account of the more complex composition of the solution, the composition of the precipitated solid is also more complex. As examples, we give in TABLE 4 the approximate compositions of the mineral in human enamel and dentin. The mineral of bone is similar to that of dentin. Both contain about twice as much magnesium and carbonate as the enamel mineral and, in addition, 1 per cent of citrate. The composition varies to some extent with the type of animal and with the diet. All of the biological apatites can be considered as being essentially hydroxyapatite containing coprecipitated secondary phosphate, carbonate, citrate, magnesium, sodium, and water.

The apatites of the enamels are, as their X-ray patterns show, fairly well crystallized. We may assume that the crystallization of the enamel apatite is a very slow process and that, under these circumstances, the coprecipitated

TABLE 4

APPROXIMATE COMPOSITION (WT. %) OF THE MINERAL SUBSTANCE IN HUMAN ENAMEL AND DENTIN

	Enamel	Dentin
Ca	37.4	35.4
Mg	.46	1.0
Na	.25	.24
P	17.4	17.6
CO_2	2.0	4.0
Cl	.3	0.
F	.02	.02

materials are distributed as individual ions throughout the apatite. They will accordingly act as substitutions in the structure and will have their effect upon the unit cell dimensions of the apatite. The *a* axis of human enamel apatite is 0.2 per cent longer than that of the standard OH apatite (TABLE 1). The enamel apatites of sharks' teeth and of elephants' molars are different from that of human teeth. Their *a* axes are 0.5 and 0.1 per cent shorter, respectively, than the *a* axis of human enamel apatite. This change in the length of the *a* axis could be explained by the substitution of OH^- by F^- and of Ca^{++} by Mg^{++}. However, the fluorine content in sharks' enamel is not high enough to be alone responsible for the change; and it has not yet been proved that the isomorphous magnesium substitution for calcium actually occurs under the conditions of the formation of biological apatites. At least, we have so far not succeeded in precipitating an apatite with shortened axes from mixed calcium and magnesium solutions. In calcite and whitlockite,[25] such a substitution does occur and is accompanied by a shrinkage of the unit cell.

The apatites of human dentin and bone are poorly crystallized. The X-ray reflections are so diffuse that the 211 and 112 reflections are not resolved and also the determination of the axis length is not better than $\pm.03$ Å. for the *a* axis (from 300) and ± 0.015 Å. for the *c* axis (from 002). On account of this comparatively low precision, the axis values do not give us any clue regarding the substitution or nonsubstitution of coprecipitant ions in the dentin and bone apatite structure. Within this precision, the axes agree with the axes of the enamel apatite. The concentration of the coprecipitants is higher than in enamel, and the apatite lattice is more distorted. Also, organic material may be occluded in the crystals, but we have no means of proving or disproving this, since the crystals, at any rate, are wholly imbedded in the organic matrix. The apatite crystals occupy about one quarter of the bone volume, while three quarters are occupied by the organic matrix (as can be calculated from the composition of bone and the densities of the components). There is enough matrix to surround each apatite crystal with a layer of matrix between one and one-half of the thickness of the crystal.

Whether the conditions of supersaturation with regard to $CaCO_3$ temporarily occur in bone or not, we cannot say. Very likely, the mechanism which allows for the comparatively fast rate of mobilization of the mineral material in the bone[26] allows also for a fast rate of deposition. If this deposition occurs rhythmically, conditions of supersaturation are more likely to occur, and layers rhythmically varying in composition would be formed. "Internal surfaces" may develop when the growth of the crystals is interrupted for some time before it continues again. But no particular enrichment of carbonate in the temporary surface is expected, unless the phosphate concentration in the solution falls below saturation or the carbonate/phosphate concentration ratio rises. That the precipitated hydroxyapatite, at least on its surface, can be decomposed again by a sodium carbonate solution, is a known fact.[27]

Conclusion. The composition and structure of the precipitates in systems as complex as the mineralizing tissues are not only determined by the composition of the solution phase of the system, but they are also materially influenced by the kinetics of the crystallizing processes. Simultaneously with

the apatite, other substances are precipitated which distort the apatite crystals and change the unit cell dimensions. A systematic study of the coprecipitation of the individual accessory components contained in the biological apatites will enlarge beyond today's status our knowledge of the structure of these apatites.

X-ray diffraction has played an important role in the elicitation of this knowledge. It has shown the essential similarities between the biological apatites and hydroxyapatite. It also has revealed characteristic differences between them and among the biological apatites themselves. It will remain an essential tool in the further studies which eventually will explain these differences in terms of composition and structure.

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LOCAL FACTORS IN THE MECHANISM OF CALCIFICATION *

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Introduction

The fact that normal mineralization takes place at specific sites in the body indicates that a "local factor(s)" present in the calcifying matrix favors the process which gives mineralized tissues their characteristic hardness. The nature and operation of the "local factor(s)" may be regarded as the key problem in studies involving the understanding of the mineralizing process.

The present approach to understanding the "local factor(s)" stemmed from physicochemical concepts of phase rule and solubility product. An attempt was made to determine from the composition and degree of mineralization whether the "local factor(s)" operates *within* these principles or in some way modifies the conditions so that the simple physicochemical principles do not apply. From both the agreements and the discrepancies between these concepts and the experimental results, further studies were designed to obtain insight into the minimal system required for the process of mineralization.

Phase Rule Principle

Composition of bone and tooth. Composition studies of the mineral portion of bone and tooth indicate wide variations. This indication is true not only for the minor components[1] such as sodium,[2, 3] fluoride,[4] and magnesium, but also for the major components,[1] particularly the ratio of phosphate to carbonate.[5-14] Our studies to date indicate that the PO_4:$2CO_3$ ratio in mineralized tissues varies from 1.88 to 10.3, as given in TABLE 1. Each figure represents the mean value for a group of animals on a given diet, and the extreme variations on either side are greater. These variations are related to fluid composition and are encountered not only in bones but in the enamel and dentin of both the growing incisors and growing molars.

The ideal distribution of two slightly soluble solids with a common ion, in this case $CaCO_3$ and $CaHPO_4$ or $CaCO_3$ and $Ca_3(PO_4)_2$, can be expressed by the following two equations:

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = \frac{KspCaHPO_4}{KspCaCO_3}\left[\frac{(Ca^{++})(CO_3^{=})}{(Ca^{++})(HPO_4^{=})}\right]^N_{sol'n} = K\left[\frac{CO_3^{=}}{HPO_4^{=}}\right]^N_{sol'n} \quad (A)$$

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = \frac{KspCa_3(PO_4)_2}{KspCaCO_3}\left[\frac{(Ca^{++})(CO_3^{=})}{(Ca^{++})^3(PO_4^{\equiv})^2}\right]^N_{sol'n} = K\left[\frac{(CO_3^{=})}{(Ca^{++})^2(PO_4^{\equiv})^2}\right]^N_{sol'n} \quad (B)$$

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TABLE 1

COMPOSITION AND CRYSTAL STRUCTURE OF MINERALIZED TISSUES

Mineral	*n = PO_4:2CO_3	X ray
Bone	1.88 — 4.24[5-10]	Apatite[15]
In vitro calc.	1.85 — 4.18[9-11]	Apatite[16]
Dentin	3.52 — 9.31[8-10, 12-14]	Apatite[13-14]
Enamel	2.08 — 10.3[8-10, 12-14]	Apatite[13-14]

* Composition = $[Ca_3(PO_4)_2]_n$. $CaCO_3$,[17] where approximately 6 per cent Ca is replaced by Mg, Na, Sr, Pb, *etc.*, and some CO_3 is replaced by citrate [18, 19, 20], PO_4 , OH, fluoride, *etc.*[10, 21]

The studies were designed to determine whether the mineral components of hard tissues, both *in vivo* and *in vitro*, obey this distribution law. For the new mineral deposited *in vitro* in rachitic bone from purely inorganic solutions, the relationship was plotted as a straight line (FIGURE 1).[9-16] The results obtained *in vivo* were plotted on the same graph and, from the present data, they also appear to have a straight line relationship. EQUATION (C) is the result. The

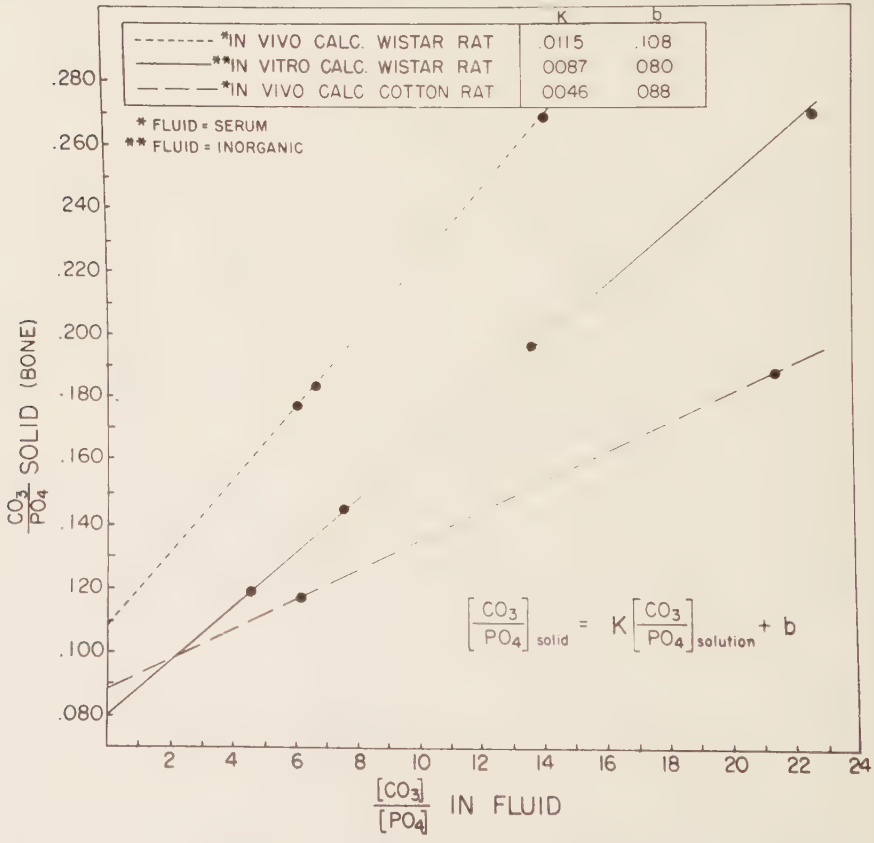

FIGURE 1. CO_3:PO_4 ratios of bone mineral calcified *in vivo* and *in vitro* in relation to fluid composition.

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = K\left[\frac{CO_3}{PO_4}\right]_{sol'n} + b \qquad (C)$$

carbonate was essentially constant in these studies. The variable factor was the phosphate in the inorganic solution for the *in vitro* studies and, in the blood serum, for the *in vivo* studies. It may be noted in FIGURE 1 that, compared to K, b is relatively constant for *in vivo* and *in vitro* calcification in the Wistar rat, as well as for *in vivo* calcification in the cotton rat. The value for b represents a constant CO_3:PO_4 ratio, probably a complex not subject to variation in this system, while K depends on a variable portion of carbonate, probably representing surface adsorption dependent on the CO_3:PO_4 ratio in solution. The carbonate in this postulated fixed complex may be part of the crystal lattice as conceived by McConnell[22] for francolite, or it could equally represent a surface compound formation in which the proportions of the combining elements are fixed by the surface area of the hydroxyapatite lattice. If b represents surface complex formation, then the larger the crystal size, the smaller should be the value of b. While no information is available about the crystal size of the mineralized tissues in these particular experiments, in the human tooth the enamel generally consists of larger crystals than bone.[23] In the cotton rat, where the molars represent teeth completely covered with enamel (unlike the incisors), b for enamel is lower than for dentin, and b is lowest for bone. To elucidate this question further, an attempt will be made to measure the actual crystal sizes in mineralized tissues obtained in such studies.

Since the fluid composition in *in vitro* calcification was under our complete control, calculations were made of the K value obtained from *in vitro* calcification data, utilizing equations A′ and B′ (below) and compared to that obtained from equations A and B.

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = K\left[\frac{CO_3^{=}}{HPO_4^{=}}\right]_{sol'n} + b, \quad \text{where } K = \frac{KspCa_3(PO_4)_2}{KspCaCO_3} \qquad (A')$$

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = K\left[\frac{CO_3^{=}}{(Ca^{++})^2(PO_4^{=})^2}\right]_{sol'n} + b, \quad \text{where } K = \frac{KspCa_3(PO_4)_2}{KspCaCO_3} \qquad (B')$$

In these calculations, the dissociation constants and activity coefficients of Hastings, Murray, and Sendroy[24] and of Sendroy and Hastings[25, 26] were employed. In addition, for the $KspCaHPO_4$, the data of Shear and Kramer,[27] and for the $KspCa_3(PO_4)_2$, the results of Logan and Taylor in the presence of small and large amounts of solid[28] were utilized. The results are given in TABLE 2. It is interesting to note that the calculated K lies between the constant for $KspCa_3(PO_4)_2$ obtained by Logan and Taylor in the presence of small amounts of solid and that obtained in the presence of large amounts of solid.[28] In making these calculations, one must bear in mind that equilibrium may not be obtained in the 18 to 24 hours employed for *in vitro* calcification. Values obtained after a period of 30 days *in vivo*, however, might well represent equilibrium. In this connection it is worth noting that the magnitude of K, in the

TABLE 2

IDEAL DISTRIBUTION CONSTANTS COMPARED TO DISTRIBUTION CONSTANTS OBTAINED IN IN VITRO CALCIFICATION

	K	
Ideal case	Equation A 68 to 70	Equation B 4×10^{-19}* 1.5×10^{-16} to 2.6×10^{-20}†
In vitro calcification	Equation A′ 0.21 to 0.25	Equation B′ 2.16×10^{-16} to 4.33×10^{-17}

* Sendroy and Hastings.[25, 26]
† Logan and Taylor.[28]

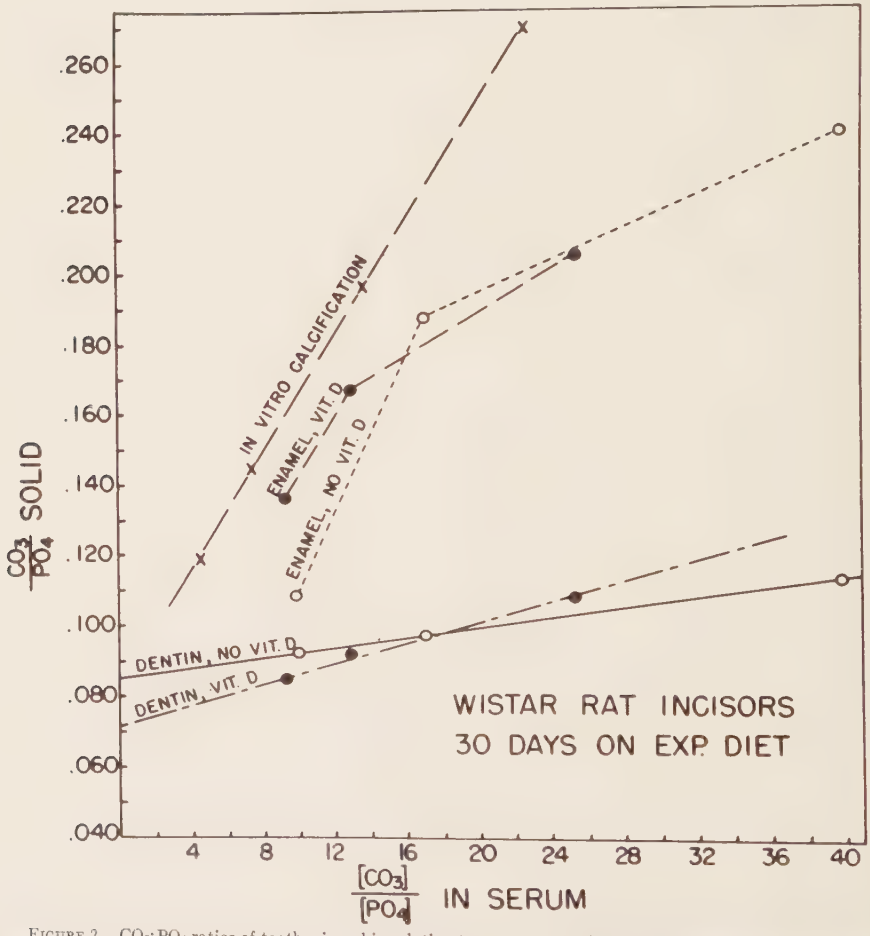

FIGURE 2. CO_3:PO_4 ratios of tooth mineral in relation to serum composition in the incisors of the Wistar rat after 30 days on experimental diets.

in vivo experiments, is sufficiently close to the results obtained in *in vitro* calcification to justify the use of *in vitro* experiments as an indication of fluid-solid composition relationships.[20] That the calculated values do not, as a rule, coincide with experimental values is indicated in the careful studies of Kolthoff and Naponen[29] of the equilibrium between mixed crystals of $BaSO_4$ and $PbSO_4$ in relation to fluid composition. The calculated constant for $\frac{KspBaSO_4}{KspPbSO_4}$ was smaller than the experimental results obtained. They found that electrolytes that do not form complexes or undissociated compounds with barium or lead do not affect the values of experimentally obtained distribution coefficients.

When the data for enamel and dentin of growing incisors of the Wistar rat[12] are plotted in the same fashion, the CO_3:PO_4 ratio of the solid is related to blood serum. This relationship is a straight line for the dentin but not for the enamel (FIGURE 2). It occurred to us that the initial amount of mineral in enamel at the beginning of the experiment, when the animal was already 23 to 25 days of age, is sufficient to mask the relationship for the growing portion of enamel. For this reason, a second study was made in which animals of the same age were kept for 45 days on the experimental diet. The results obtained in this experiment can be considered a straight line, as seen in FIGURE 3. It is interesting to note that, while vitamin D influences the K value (FIGURES 2, 3), representing the relation between blood and mineral composition, it does not influence the

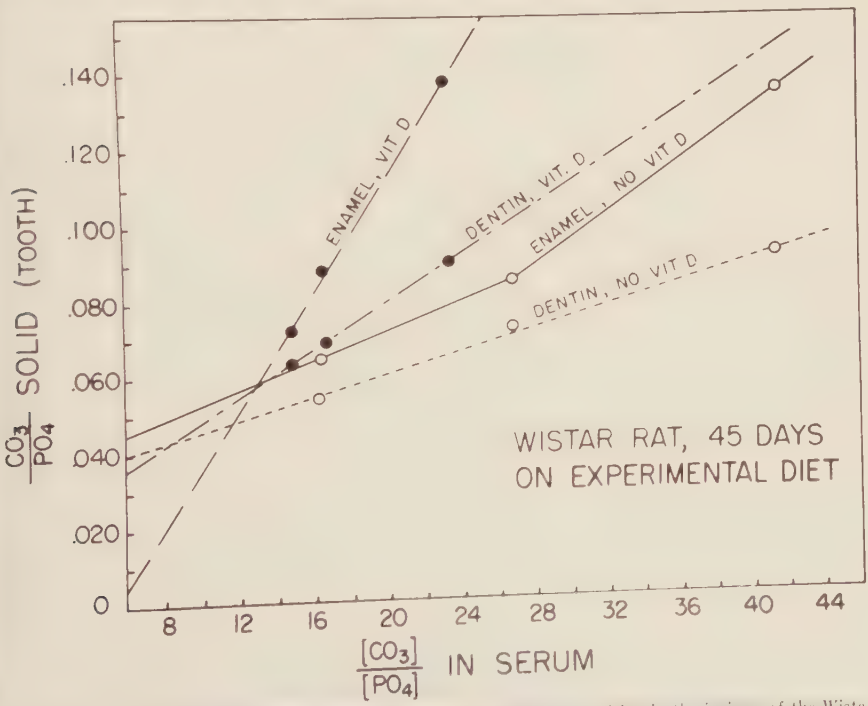

FIGURE 3. CO_3:PO_4 ratios of tooth mineral in relation to serum composition in the incisors of the Wistar rat after 45 days on experimental diets.

percentage of mineralization.[12] This effect is in marked contrast to our findings in bone, where, to the degree that vitamin D raised the Ca × P product, it markedly influenced the degree of mineralization as expressed by per cent ash.[6, 7] From this finding *it is concluded that, during the relatively short time that the tooth matrix can undergo active mineralization, the activity of the local factor(s) is so high that maximal mineralization takes place at much lower products of calcium times phosphate ions than is the case for bone at the corresponding age of the animal.* It is known from *in vitro* calcification studies[10] that embryonic bone begins calcifying at a serum Ca × P of 16, whereas with the older rachitic bone, the minimal serum Ca × P is about 35. Thus, embryonic bone would indeed mineralize well under conditions where older rachitic bones would not.

These studies indicate that the composition of the mineral deposited is influenced by the composition of the fluid from which these precipitates form in all three mineralized tissues, namely bone, dentin, and enamel, as would be expected from phase rule considerations. The exact nature of this relationship is determined by the "local factor(s)," as shown by the differences in composition of bone, dentin, and enamel in the same animal. The "local factor(s)" may play a part in the fact that, despite wide variations in composition, the crystal structure of such minerals, both in new and old calcification, is predominantly apatite. Moreover, "local factors" influence the degree of mineral-

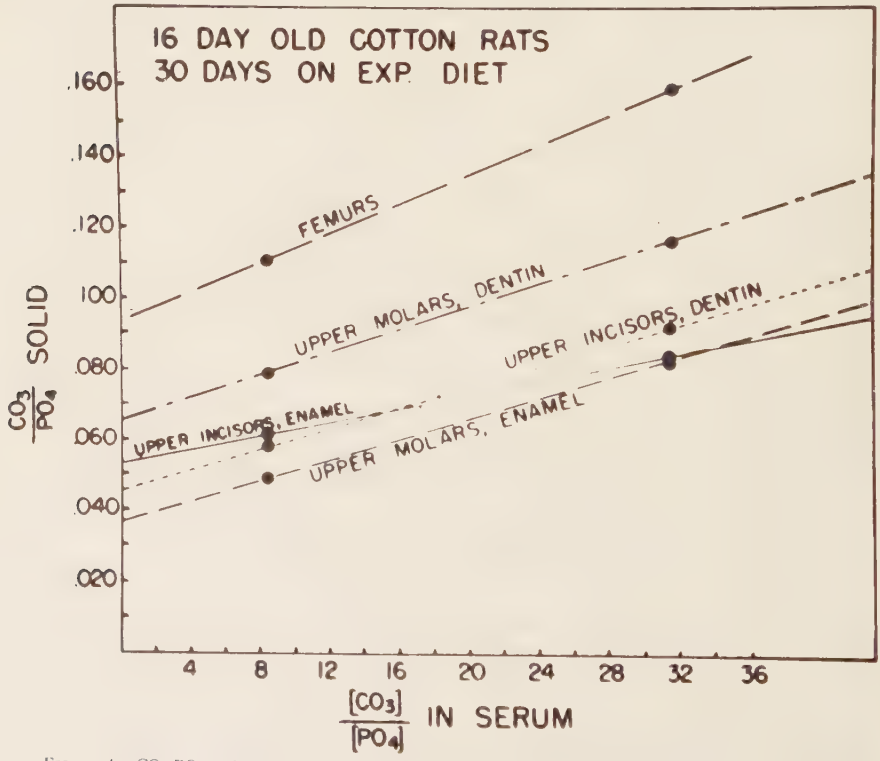

FIGURE 4. CO_3:PO_4 ratios of the bones and teeth of the cotton rat in relation to serum composition.

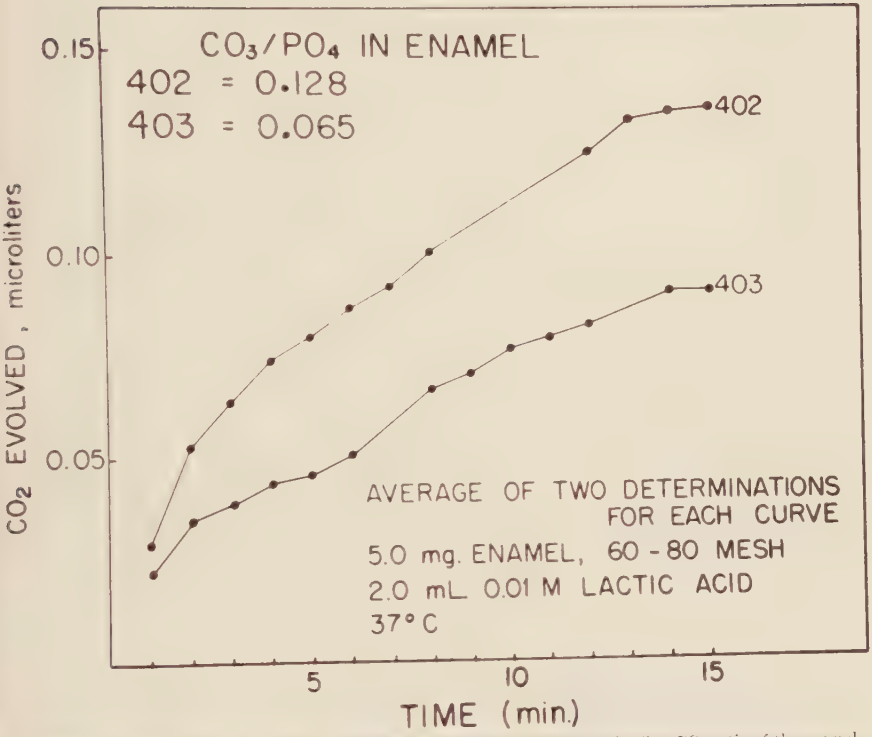

FIGURE 5. Carbon dioxide evolution in acid-treated enamel in relation to the CO_3:PO_4 ratio of the enamel.

ization from a given fluid, as indicated by the fact that vitamin D does not influence the degree of mineralization in the teeth under conditions where it does influence the degree of mineralization in bone.

Caries susceptibility and composition of bones and teeth. The possibility that caries susceptibility is related to high carbonate tooth composition is indicated in the working hypothesis[12, 30] given below:

(a) Acids dissolve carbonate preferentially from bone and tooth mineral.[31-35] That carbonate dissolves more rapidly from high carbonate enamel and dentin is illustrated in FIGURES 4 and 5.[36]

(b) $Ca_3(PO_4)_2$ is more soluble in the presence of carbonate due to the formation of unionized carbonato-phosphate complexes.[37, 38]

For these studies, the cotton rat was chosen because one can produce caries in this species resembling those of human teeth. The problem was: can one develop experimental conditions for controlling the composition of the molars? Unlike the continually growing incisors and bones, once mineralization is complete in the molars, exchange with body fluids takes place to a limited degree only. If one is to control the molar composition, the body fluid composition must be regulated early in the life of the animal, before mineralization of the molars has taken place. Ideally, the animals should be subjected to experimental diets immediately after birth. The young rats were actually placed

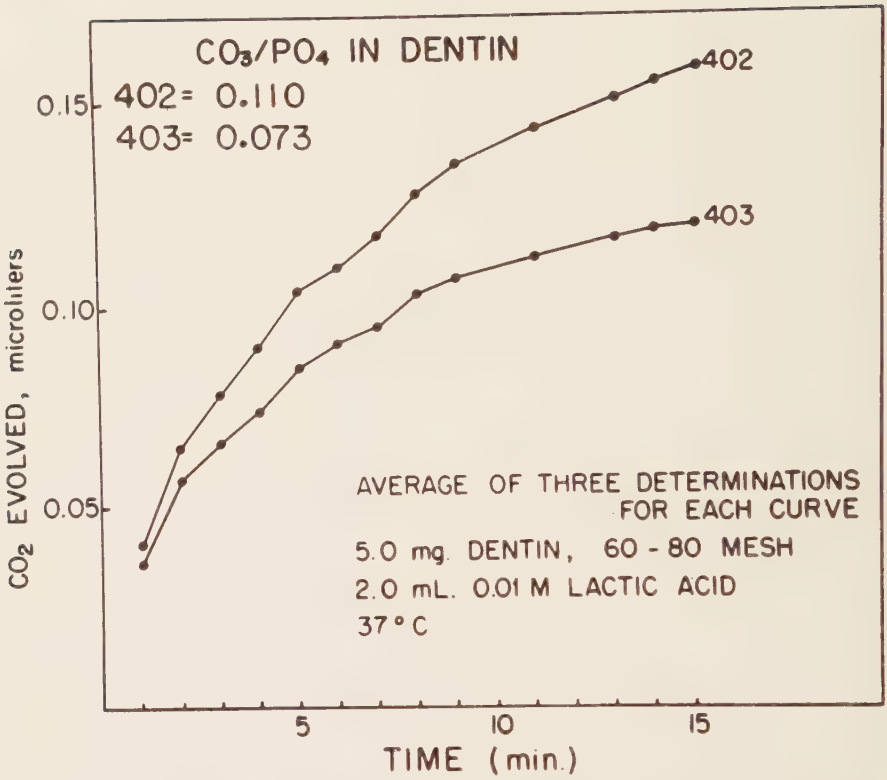

FIGURE 6. Carbon dioxide evolution in acid-treated dentin in relation to the CO_3:PO_4 ratio of the dentin.

on these diets at 16 days of age, the earliest age at which they could be safely weaned from the mothers. At this time (according to information from Doctor James H. Shaw of the Harvard School of Dental Medicine), the first molars are calcified, the second molars are about half calcified, and the third molars are not yet mineralized. FIGURE 6 illustrates that, in the cotton rat, the inorganic composition of bone and of tooth enamel and dentin in the growing incisors and molars is related to blood composition. The blood CO_3:PO_4 ratios were influenced by dietary Ca:P ratios, just as in the case of the Wistar rat.[6-10, 12-14] That caries susceptibility is related to high carbonate teeth is shown in TABLE 3. The caries index evaluation was done by Doctor James

TABLE 3

INFLUENCE OF DIET ON CARIES SUSCEPTIBILITY IN THE COTTON RAT

Diet	Type of tooth	Number of animals	Number of carious lesions	Extent of carious lesions
High Ca, low PO_4	High CO_3	76	10.9	26.0
Low Ca, high PO_4	Low CO_3	57	5.5	14.2
"P"			<0.01	<0.01

H. Shaw. These results probably underestimate the real situation because (a) we obtained a few litters with no difference in caries susceptibility where we had reason to believe that other factors such as infection entered. We decided, nevertheless, to include all data consecutively; and (b) at 16 days of age, the first molar of the cotton rat is almost completely mineralized, and thus the influence of the diet on composition is not as great as would be the case if we had the animal's blood under our control from its day of birth. Still, the difference is impressive, and indicates that the working hypothesis is valid and that high carbonate teeth are more caries-susceptible.

Inherent Variations in the "Local Factor(s)"

In these studies, differences in the composition of bone, dentin, and enamel in a single animal indicate that there are hereditary variations in the "local factor(s)." Hereditary differences in the functioning of the "local factor(s)" are also manifest when one compares results obtained in the cotton rat to those in the Wistar rat. In the Wistar rat the bone PO_4:$2CO_3$ is lower, and the Ca:P is higher than in the cotton rat on identical diets.[39] Whether we are dealing with distinct entities of the "local factor(s)" or simply modifying influences on the system responsible for mineralization (such as different auxiliary enzymes or changes in fluid composition, due to differences in the transportation system from the blood serum to the site of mineralization), cannot be evaluated at present. It can be stated, however, that these differences represent modifications in the "local factor(s)" inherent in the different tissues or species.

Even though, at present, the exact reasons for the differences in the "local factor(s)" cannot be defined, it can be stated that variations in the CO_3:PO_4 in the fluid (serum) will cause variations in the same direction in the CO_3:PO_4 ratio of the solid. The nature of the relationship between the fluid and solid appears to be defined by hereditary factors.

Solubility Product Principle and Calcification

Studies of *in vitro* calcification show the operation of the solubility product principle in that new mineralization from a purely inorganic solution depends on concentrations of calcium and phosphate ions in excess of a critical product. The operation of the "local factor(s)" is indicated by the variations of this minimal Ca × P product, which depends on the type of bone, as shown in TABLE 4. For embryonic bone,[40] Ca × P = 16, for the usual rachitic bone,[41-43] it is 35, in beryllium rickets,[44] 60, and in strontium rickets 90.[43, 45, 46]

TABLE 4

THE SOLUBILITY PRODUCT PRINCIPLE AND NEW CALCIFICATION

Bone	Ca × P*	Reference
Embryonic	16	40
Rachitic	35	41, 42, 43
Be rickets	60	44
Sr rickets	90	43, 45, 46

* Minimum for new calcification *in vitro*; Ca and P are expressed as mgm. per cent; P represents inorganic phosphate.

These observations suggest that while the solubility product principle operates for new mineralization, the availability of some factor(s) at the specific site of mineralization is equally important.

Reversible Inactivation of Calcification in Vitro

Further studies of strontium rickets led to experiments in which it was possible to demonstrate the reversible inactivation of calcification *in vitro*. When strontium carbonate replaces calcium carbonate in a rickets-producing diet, rickets develops which is histologically similar to calcium rickets, but which does not respond to vitamin D. Furthermore, such rachitic cartilage does not calcify under conditions suitable for calcification *in vitro* of the control cartilage. When strontium carbonate is replaced by calcium carbonate in the diet, the bones heal *in vivo* with vitamin D and *in vitro* in calcifying solution, in a manner similar to that of the control rachitic bone. Moreover, strontium rachitic bones, after shaking with calcium chloride, will calcify *in vitro* in a manner similar to control rachitic bones (referred to as calcium rachitic bones).[10, 43, 45-47]

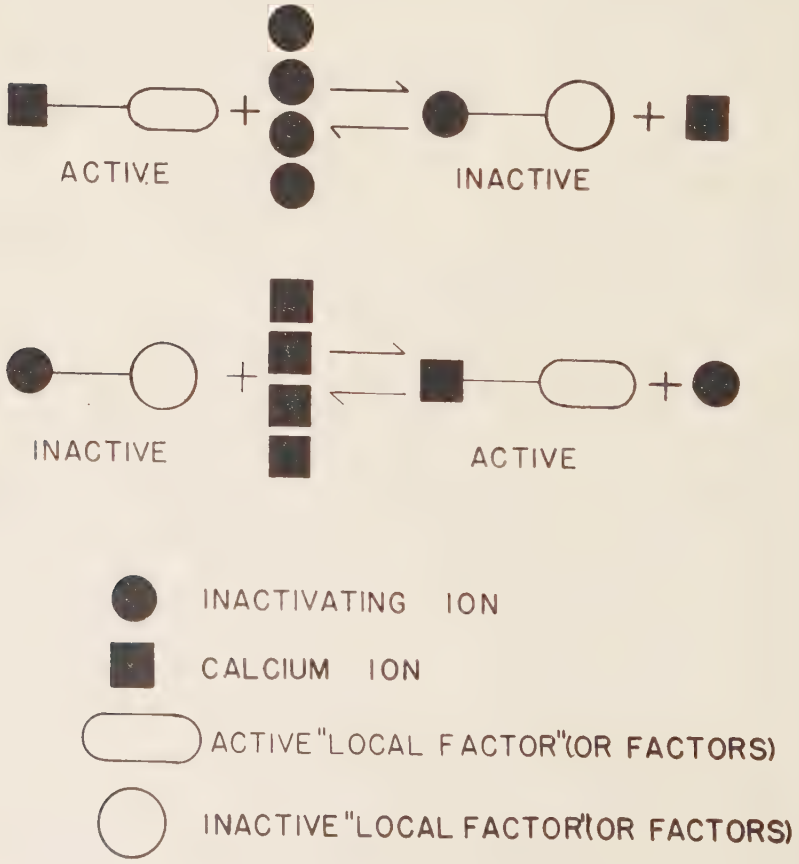

FIGURE 7. Reversible inactivation of calcification *in vitro* (tentative mechanism).

The observations made with respect to the survival of the calcifying mechanism are summarized as follows:

Strontium rachitic bone sections do not calcify after 18 hours in a solution with Ca × P = 60 (Ca = 10 mgm. per cent; P = 6 mgm. per cent), and Mg = 0.75 mM per liter. After 48 additional hours in the same solution, however, the calcification is similar to that of control calcium rachitic bone sections after 18 hours in the same solution.

Control sections do not calcify when kept for 18 hours in a solution with a low Ca × P product of 20 (Ca = 10 mgm. per cent; P = 2 mgm. per cent). Such sections will not calcify *in vitro* in the next 48 hours when transferred to the solution with the higher Ca × P = 60, and Mg = 0.75 mM per liter.

The studies with strontium rickets were in harmony with the concept that strontium combines reversibly with a constituent in the calcifying tissue essential to calcification.[43, 45-47] This finding was further indicated by the fact that it is possible to deposit strontium *in vitro*.[48, 49] If this concept is correct, it it should be possible to inactivate the calcifying mechanism reversibly in the usual type of bone, as shown in FIGURE 7.

In experiments based on the above hypothesis, it was possible to inactivate and reactivate the calcifying mechanism. The order of the inactivating power of the ions is $Be^{--} > Cu^{--} > Mg^{--} > Na^{+} > Sr^{++} > K^{+}$ (TABLE 5). This order is in the same direction as the reciprocal of the ionic radii.[50] That reactivation is not due simply to the presence of calcium ions in solution is evident from the fact that inactivation takes place in the same concentration of calcium ions as does the reactivation, the only difference being a relatively small amount of inactivator. These results indicate that a step in calcification is the combination of calcium ions with some constituent of the bone tissue,

TABLE 5

THE REVERSIBLE INACTIVATION OF CALCIFICATION IN VITRO

Experiment	Treatment	Degree of calcification† (mean)
Control	Rachitic bone section placed in calcifying solution.*	2^{++++}
Inactivation	Rachitic bone section shaken for two hours with the chloride salts of the cation mixtures shown below, then washed with distilled water, and then placed in calcifying solution. (1) 150 mE/L Ca^{++} and 0.1 mE/L Be^{++} (2) 150 mE/L Ca^{++} and 0.5 mE/L Cu^{++} (3) 150 mE/L Ca^{++} and 10 mE/L Mg^{++} (4) 150 mE/L Ca^{++} and 50 mE/L Na^{+} (5) 150 mE/L Ca^{++} and 100 mE/L Sr^{++} (6) 150 mE/L Ca^{++} and 400 mE/L K^{+}	0^{0} or 1^{+}
Reactivation	Rachitic bone section shaken for two hours with the chloride salts of the cation mixtures shown above, then washed with distilled water, then shaken for one hour with 150 mE/L Ca^{++} as the chloride salt, and finally placed in calcifying solution.	3^{++++} to 4^{++++}

* Calcifying solution per liter: 97 mM Na^{+}, 5 mM K^{+}, 75 mM Cl^{-}, 22 mM HCO_3^{-}, 25 mM Ca^{++} (10 mgm. per cent), and 1.6 mM $PO_4^{\equiv}$ (5 mgm. per cent as P); pH 7.3; temperature 37° C.

† For the scale used in evaluating the degree of calcification, see Sobel[10] and Sobel *et al.*[44]

TABLE 6
INFLUENCE OF Be:Ca RATIOS ON INACTIVATION AND REACTIVATION

Inactivation			Reactivation	
$BeCl_2$ solution	$CaCl_2$ solution	Degree of calcification†	$CaCl_2$ solution	Degree of calcification†
(*mEq/L*)	(*mEq/L*)	(*mean*)	(*mEq/L*)	(*mean*)
0.1	50	0^0	150	4^{++++}
0.1	150	1^{++++}	150	2.5^{++++}
0.1	300	4^{++++}	150	4^{++++}
1.0	50	0^0	150	2^{++++}
1.0	150	1^+	150	1.5^{++++}
1.0	300	2^{++++}	150	3.5^{++++}
10.0	50	0^0	150	0^0
10.0	150	0^0	150	0^0
10.0	300	0^0	150	1^+

Ca × P product = 50. Degree of calcification of untreated control (mean) = 2^{++++}.
† For the scale used in evaluating the degree of calcification, see Sobel[50] and Sobel *et al.*[51]

probably part of an enzyme system,[51, 52] before mineralization can take place. This inactivation is a function of the inactivator to calcium ratio, as shown for beryllium and calcium in TABLE 6.[53]

Chondroitin Sulfate and the Calcifying Mechanism

The possible involvement of a mucopolysaccharide, almost certainly chondroitin sulfate,[54] in the calcifying mechanism was proposed as an explanation of the reversible inactivation of calcification *in vitro*. Heated bone sections, later treated with calcium ions, still retained their ability to calcify. While heating destroys enzymes in the phosphorylative cycle, chondroitin sulfate still remains, as shown by metachromatic staining.

An evaluation of the literature is also in harmony with the concept that chondroitin sulfate may be an integral part of the calcifying mechanism. Sulfate-containing mucopolysaccharides appear whenever calcification takes place, namely, in the dentin and the enamel,[55] in the bone,[56] and in abnormal calcification of the arteries.[57] The presence of metachromatic staining has been indicated in various types of pathological calcification, *i.e.*, calcinosis universalis, calcified bursitis, and renal stones of the calcium, phosphate, carbonate type.[58] Significantly, no indication of mucopolysaccharides was detected in calcium oxalate and ammonium urate stones.[58] Based on the inhibition of calcification *in vitro* of rachitic cartilage by toluidine blue and other basic and acid dyes, it has been suggested that chondroitin sulfate may be essential for the calcification of hypertrophic rachitic cartilage.[59] Under certain conditions, collagen will react with chondroitin sulfate so vigorously that sulfate will replace absorbed dyes from the collagen.[60] Hyaluronic acid and other mucopolysaccharides will not have this effect. Since collagen is the key protein of bone and dentin, these experiments again suggest that the affinity of collagen for chondroitin sulfate may be one of the properties of the protein which produces the combination responsible for initiating calcification. Calcium is particularly effective in causing a shortening of the length of collagen fibers, with a

subsequent release of chondroitin sulfate.[61] Calcium ion increases the viscosity of at least some mucopolysaccharides, thus indicating polymerization.[61] This polymerization, at least for hyaluronate by means of calcium ion, was related to the rate of prothrombin conversion to thrombin.[62]

The role of chondroitin sulfate as an integral part of the "local factor(s)" is further suggested by experiments with toluidine blue and protamine, where the inactivation again is a function of the inactivator to calcium ratio[63-65] (TABLES 7 and 8). In the case of these inactivators, unlike the inactivation with mineral cations, a more specific target, namely chondroitin sulfate can be proposed.

TABLE 7

INFLUENCE OF TOLUIDINE BLUE "O" OR PROTAMINE TO CALCIUM RATIOS ON SUBSEQUENT CALCIFICATION IN VITRO

Inactivation			Reactivation with 150 mEq/L $CaCl_2$
Toluidine blue "O" solution	$CaCl_2$ solution	Degree of calcification†	Degree of calcification†
μM/L	*mEq/L*	*Mean*	*Mean*
39	0.0	0^0	1.2^{++}
39	25.0	1^+	2.2^{++}
39	150.0	1.8^{+++}	3^{+++}
39	100.0	1^{+++}	
1	139.0	1.3^{++++}	
0.6	144.0	1.7^{++++}	
0	0.0	0^0	
0	150.0	2^{++++}	
Protamine sulfate solution	$CaCl_2$ solution	Degree of calcification*†	Degree of calcification*†
per cent	*mEq/L*	*Mean*	*Mean*
0.4	0.0	0^0	1.3^{++}
0.4	5.0		2.5^{++}
0.4	10.0	1.5^+	
0.4	25.0		2.3^{+++}
0.4	150.0	1^{++}	3^{+++}

* Surface covered, calcification was below the surface, became evident on clearing the sections.
† Degree of calcification of untreated controls was 2 [illegible] mean . For scale used in evaluating degree of calcification, see Sobel[59] and Sobel *et al.*[44] Following preliminary soaking in toluidine blue "O" $CaCl_2$ solutions for one hour, and protamine-$CaCl_2$ solutions for two hours, sections were placed in calcifying solution for 18 hours at 37° C., pH 7.3 ± 0.07. Ca = 10 mgm. per cent; P = 5 mgm. per cent.

TABLE 8

CALCIFICATION IN VITRO IN THE PRESENCE OF TOLUIDINE BLUE "O"

T.B.O*	Degree of calcification†	Degree of metachromasia
μM/L	*Mean*	*Mean*
15.8	0	doubtful
5.3	1.0^{++++}	doubtful
2.2	1.2^{++++}	0
1.7	1.0^{++++}	0
0	2.3^{++++}	—

* T.B.O = toluidine blue "O."
T.B.O was placed in the calcifying solution. Ca = 10 mgm. per cent; P = 5 mgm. per cent; at 37° C. for 18 hours; pH 7.3 ± 0.07.
† For scale used to evaluate the degree of calcification, see Sobel[10] and Sobel *et al.*[44]

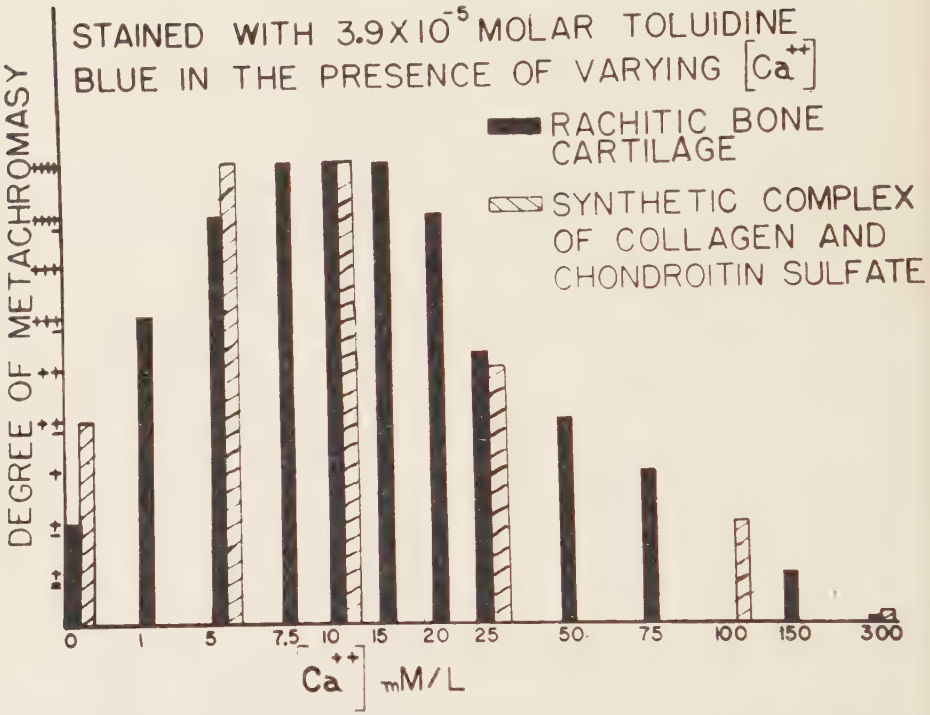

FIGURE 8. Influence of calcium ion on the degree of metachromasy of rachitic bone cartilage and synthetic collagen-chondroitin sulfate complex.

In attempting to relate the "local factor(s)" to the state of polymerization of chondroitin sulfate, the influence of calcium ions on metachromatic staining was investigated. With a constant amount of toluidine blue, the degree of metachromasia increases with calcium ion concentration in solution, up to a maximum of about 15 mEq. per liter (FIGURE 8). Above this concentration of calcium ions, there is a gradual decrease in metachromasia. Prior shaking with calcium chloride increases the intensity of metachromatic staining in the ossifying matrix. In contrast, when chondroitin sulfate is extracted from bone, calcium ion competitively interferes with metachromatic staining. However, the metachromasia obtained with isolated chondroitin sulfate combined with isolated collagen responds to calcium ion in the same way as rachitic bone cartilage (FIGURE 8). When this complex is first shaken with calcium ions and then with phosphate a silver stain typical of *in vitro* calcification is obtained. Moreover, when this complex is placed in solutions employed for *in vitro* calcification, there is an increase in ash compared to control specimens of collagen or chondroitin sulfate similarly treated.

From the results obtained, the process visualized is that of calcium and toluidine blue competing for the chondroitin sulfate complex. Calcium ion, up to a certain concentration, causes a rearrangement of the chondroitin sulfate complex, so that it becomes more active metachromatically, probably due to a

changed state of polymerization. After reaching this new configuration, with further increased concentrations of calcium ions, competitive behavior becomes evident by decreased metachromasia, since the calcium binding is no longer compensated by further intensification of metachromatically active changes in the molecule.

The property of calcium ions increasing the degree of metachromasia seems to be typical of calcifying cartilage in the studies carried out to date. The appearance of metachromasia in bone cartilage did not correlate in all cases with calcifiability (TABLE 9). However, metachromasia was not enhanced by calcium ions when calcifiability was destroyed and metachromatic activity still survived. Bones examined after treatment (TABLE 9) in basal salt solution, in formaldehyde, or in 95 per cent phenol showed maximal metachromasia in the absence of calcium ions (1 mEq. per liter in the case of phenol). The degree of metachromasia decreased at 20 mEq. per liter and disappeared at about 50 mEq. per liter (see FIGURE 9). These findings suggest an alteration in the

TABLE 9

METACHROMASIA AND CALCIFICATION BY VARIOUS TREATMENTS[65]

Treatment	Before shaking with $CaCl_2$		After 1 hr. shaking with 150 mEq/L $CaCl_2$	
	Metachromasia‡	Calcification	Metachromasia‡	Calcification
Shaken 2 hr. in basal salt sol'n at 37° C.	+++	1^{++++}		
Shaken 6 hr. in basal salt sol'n at 37° C.	++	0		
Shaken 2 hr. in basal salt sol'n containing 5 mEq/L Ca^{++}, 37° C.	++++	2^{++++}		
Shaken 6 hr. in basal salt sol'n containing 5 mEq/L Ca^{++}, 37° C.	++++	2^{++++}		
Shaken 1 hr. in distilled water at room temperature	±	0	+++	2.5^{++++}
Heated 30 min. in distilled water at 97° C.	++	0	+++	2^{+++}*
Shaken 1 hr. in xylene	±	0	++±	2.5^{++++}*
Shaken 1 hr. in ethyl alcohol, 95%	++	1^{+}		2^{+++}
Shaken 1 hr. in ethyl alcohol, 95% + solid $CaCO_3$		2^{+++}		4^{++++}
Shaken 1 hr. in 38% formalin	++++†	0	++++†	0
Shaken 1 hr. in methyl alcohol, absolute	+++†	0	+++†	0
Shaken 1 hr. in 95% phenol (95 gm. phenol + 5 gm. water)	+	0	+++†	0

* See Sobel and Hanok[65], table VII.

† Metachromasia was extremely intense and appeared as a velvety, bronze colored structure. Microtomed sections, viewed by transmitted light, appeared magenta colored

‡ For scale used in evaluating the degree of metachromasia, see TABLE 9(A).

TABLE 9(A)

The degree of metachromasia is graded as follows:

0 = no purple (metachromatic) stain.

± = broken thin purple line extending latitudinally along the epiphyseal cartilage plate.

+ = continuous thin purple line extending along the epiphyseal plate.

++ = continuous purple line extending longitudinally half way up the epiphyseal plate.

+++ = continuous purple line extending longitudinally three quarters of the way up the epiphyseal plate.

++++ = complete purple stained epiphyseal cartilage.

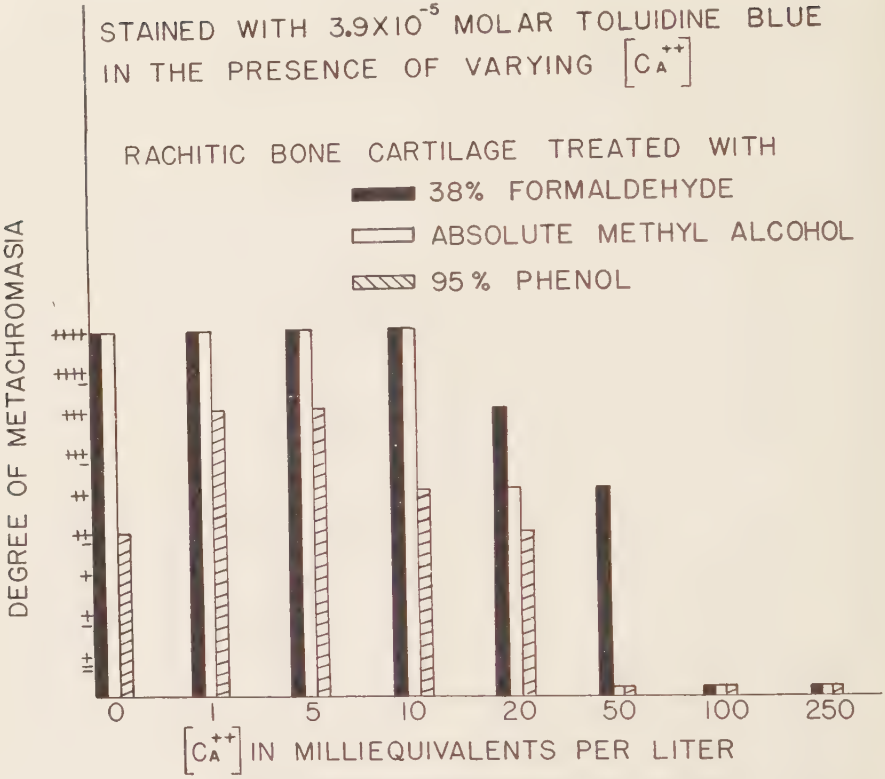

FIGURE 9. Influence of calcium ions on metachromasia in cartilage with destroyed calcifying mechanism.

binding of chondroitin sulfate. A possible explanation is that some of the chondroitin sulfate is freed of the protein bond.

In future studies, chondroitin sulfate prepared from various sources will be condensed with collagen prepared from various tissues by several methods. These collagen-chondroitin sulfate complexes will be examined by methods analogous to *in vitro* calcification to determine which of these may be responsible for the formation of bonelike minerals. If these experiments are successful, an attempt will be made to condense keratin (the key protein of enamel) with chondroitin sulfate to determine whether these complexes may initiate production of mineral similar in structure to that found in enamel. With all the above compounds, the influence of calcium ion on the degree of metachromasia will be investigated and, if possible, used as a guide to mineralization.

Summary

Studies of *in vivo* and *in vitro* calcification indicate that the relation between fluid and solid composition of the mineralized tissues can be expressed by the equation:

$$\left[\frac{CO_3}{PO_4}\right]_{solid} = K\left[\frac{CO_3}{PO_4}\right]_{sol'n} + b$$

where b is visualized as a constant factor in composition and K represents the variable influenced by the fluid CO_3:PO_4 ratio. Evidence is presented to indicate that the "local factor(s)" of calcification defines the values of b and possibly K.

Comparison of the mineral composition of enamel, dentin, and bone, and, moreover, comparison of composition of these tissues in the cotton rat and the Wistar rat, indicate inherent variations in the manner in which the "local factor(s)" operates.

Studies of *in vitro* calcification indicate that there is a minimal product of calcium and phosphate ions required for new mineralization. The magnitude of this minimum varies and depends on the operation of the "local factor(s)."

Studies derived from the reversible inactivation of calcification *in vitro* indicate that a complex of chondroitin sulfate with collagen in a critical configuration may be responsible for initiating the process of calcification in preosseous cartilage.

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Discussion of the Paper

Doctor Leonard F. Bélanger (*University of Ottawa, Ottawa, Ont., Canada*): After Doctors Sobel and Burger reported at the International Physiological Congress in Montreal (1953) that they considered chondroitin sulfate as presumably the "local factor" favoring mineralization in their experiments on cartilage, an attempt was made to identify possible organic-bound calcium in the epiphyseal plate by obtaining *in vitro* exchange of this calcium in sections with ionized Ca^{45}. The procedure was essentially that described in the *Journal of Dental Research* (1953. **32:** 168). Acid demineralized sections of the tibia of 200 gram rats were placed in a weak solution of Ca^{45} for two hours and afterwards washed thoroughly in distilled water, dehydrated in graded alcohols, and covered with a thin coat of celloidin and dried. Autoradiographs from such sections have revealed the presence of Ca^{45} in the epiphyseal plate with progressively increasing concentrations from the formative portion to the more advanced regions where calcification takes place. In these autoradiographs no

picture was detected from the articular cartilage or over the bone itself. Similar sections incubated for six hours in 1 per cent hyaluronidase at 37° C. showed approximately ⅓ Geiger counts obtained from the precedent group and no autoradiographic record for an exposure of nine days similar to that of the first group. It would thus appear that there are present in the cartilage of the epiphyseal plate substances labile in hyaluronidase and also metachromatic, presumably sulfopolysaccharides which have the ability to adsorb or exchange calcium *in vitro* (Anat. Record. In press).

EFFECT OF NUTRITIONAL FACTORS ON BONES AND TEETH

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Introduction

Numerous essential nutrients are directly involved in the development and maintenance of the calcified tissues. It is the purpose of this review to discuss these nutritional influences in terms of the effects upon the elaboration of the organic matrix in bones and teeth, the process of calcification, and the biochemical processes involved therein. During the preparation of this review, I have become fully cognizant of the many unexplored areas in histochemical and biochemical knowledge for which answers are needed to supplement the precise morphological descriptions of nutritionally-induced abnormalities. Throughout, I have been challenged by the many possibilities for collaboration between the biochemist, the radiobiologist, and the histochemically-minded pathologist.

For the sake of completeness and integrated approach, and in view of the fact that this area has not been reviewed recently, an appreciable amount of textbook material has been included with respect to the morphological changes and the biochemical alterations in vitamin and mineral deficiencies. Frequently, these changes can be used as valuable experimental tools for the exploration of the fundamentals of structure, composition, and growth of the mineralized tissues.

Vitamin A Deficiency

General. In the deficiency of vitamin A, the most prominent abnormality is the characteristic metaplasia observed in the epithelial structures. These changes occur before the rate of growth in young animals is materially affected and appear to be specific primary consequences of vitamin A deficiency. Epithelia of ectodermal, mesodermal, and entodermal origin are influenced in a similar fashion. Wolbach and Bessey[1] have described the pathology of the epithelial lesions as an "atrophy of the epithelium concerned, reparative proliferation of basal cells and growth and differentiation of the new products into a stratified keratinizing epithelium." These investigators stated that these specific changes were found in those epithelia whose cells "have a secreting (chemical) function in addition to the role of a covering layer and whose functioning cells are without power to divide."

Repair is believed to be initiated by the lowermost cells of the replacement epithelium which have proliferative powers, as in the stratum germinativum of the epidermis. This replacement epithelium is identical in all locations regardless of the original function and structure of the tissue. This layer proliferates and differentiates into epithelium which is characteristic of the region, whereas the layers above, which were irreversibly destined to keratinization, become vacuolated, invaded by polymorphonuclear leukocytes, and disappear.

Actually, these epithelial changes in avitaminosis A and recovery therefrom are closely similar to, if not identical with, those changes which occur in the vaginal epithelium during the estrus cycle of mammals. It is noteworthy that the epithelial changes occur in similar fashion, although to varying degrees, irrespective of the embryonic origin of the epithelium.

Teeth. Because of the epithelial origin and the secretory function of the ameloblasts, it is not surprising to find that the deficiency of vitamin A profoundly affects their development, differentiation, and functional influence. In the normal growth of the rodent's continuously growing incisor, the following orderly sequence may be observed: (1) proliferation of ameloblasts; (2) differentiation of ameloblasts; (3) differentiation of odontoblasts; (4) formation of dentin matrix; (5) formation of enamel matrix; and (6) calcification of dentin and enamel matrices. Throughout life, the odontogenic epithelium remains active as a tooth organizer and also in the formation of enamel. On the labial side, this layer of cells extends the full length of the unerupted portion of the incisor. On the lingual side, it extends a very short distance and acts only as an organizer.

The underlying changes reported by various investigators[2-5] in the incisors of vitamin A-deficient rats and guinea pigs are in good agreement and appear to be well established. The first histologically visible abnormality in the teeth is observed in the odontoblasts. There is a failure of the mesenchymal pulp cells to differentiate into functional odontoblasts and an incomplete arrangement of the latter into the characteristic alignment. The odontoblasts show varying degrees of development in various areas of the teeth, being more poorly differentiated proximally than distally. This variation is probably due to the fact that the former cells are younger and hence more likely to be deficient than those which were differentiated more distally and, therefore, earlier. This dedifferentiation occurs while the odontogenic epithelium is still being proliferated in morphologically normal fashion. In consequence of the abnormalities in the odontoblastic layer, dentin formation is irregular and in varying amounts. The lingual (cementum-covered) dentin is thin, while that deposited over the labial surface (enamel-covered) is thicker than normal.

Since the odontoblasts are organized by the odontogenic epithelium, this failure of the odontoblasts to differentiate would appear to be due to inadequate physiological stimuli from the ameloblasts, even though the latter appear to be normal as judged by the usual morphological criteria. Thus the primary consequence of vitamin A deficiency is evidently upon the odontogenic epithelium.

Later, in the deficiency, profound anatomical changes in the ameloblasts are observed. These cells exhibit such a lack of differentiation that virtually no recognizable ameloblasts can be found. Consequently, there is a great reduction in the deposition of enamel matrix. As a result, enamel hypoplasia is a prominent manifestation of advanced vitamin A deficiency. It is noteworthy that the odontogenic epithelium in the rodent does not stop its proliferative activity. This results in the invasion of cords of undifferentiated epithelium into the pulpal tissues to form nests of cells. Some of these undifferentiated cells still have the ability to stimulate the neighboring mesenchyme. Abortive

attempts at dentin formation occur which result in numerous irregular concretions within the pulp.

Repair patterns in the rodent recovering from vitamin A deficiency are straightforward and rapid. The odontogenic epithelium regains its function and morphological appearance. This reorientation is followed by the formation of normal odontoblasts and the deposition of normal dentin. Where there has been an infolding of the odontogenic epithelium, recovery often results in tooth duplications and tumor-like formation.[6]

Changes of a comparable nature in the developing teeth of vitamin A-deficient infants have been reported by Boyle[7] and by Dinnerman.[8]

Bone, postnatal. In the early studies of vitamin A deficiency, Wolbach and Howe noted an impairment of epiphyseal bone formation.[2] They interpreted this to be a manifestation of the degree of inanition occasioned by the vitamin A deficiency. Later, it became evident that impairment of epiphyseal bone growth was a primary result of the general deficiency syndrome, since it occurred early in the production of the deficiency before the cessation of over-all growth. In other studies, the statement has been made that vitamin A deficiency is the only means of causing severe retardation of skeletal growth prior to appreciable growth retardation in the soft tissues.[9, 10]

In about the same era, neurological signs together with lesions in the nervous tissues were reported from a number of laboratories as important entities of a primary nature in the syndrome of experimental vitamin A deficiency. Mellanby observed evidence of degeneration in the cranial and peripheral nerves, together with similar lesions in the gray and white matter of the brain and spinal cord.[11] Even though there was no agreement as to the pathogenesis or the distribution pattern of the neurological lesions, vitamin A was postulated to have a specific effect on the maintenance and development of the nervous tissues.

Thus there were two schools of thought: the one in which impaired epiphyseal bone growth was believed to be primary and the nerve lesions secondary; and the other in which the lesions in nervous tissues were believed to be primary manifestations of vitamin A deficiency. The controversy was largely resolved by the statement by Wolbach and Bessey that "the genesis of the nerve lesions of vitamin A deficiency requires an essentially normal rate of growth of a normal nervous system and that mechanical injury, the result of a disproportion between the central nervous system and its bony enclosure, is the explanation."[10] This interpretation was based on several important observations of which the foremost was that the normal lesions have been observed only in young, actively growing animals. Rats placed on a vitamin A-deficient regimen after growth had ceased, failed to develop neurological manifestations. In addition, the pattern of the lesions was unpredictable, either in a single animal or in comparing several animals from a given group with each other. Careful dissection of the nervous tissues indicated that the cerebellum herniated into the foramen magnum. Multiple herniations of the cerebrum and cerebellum into the dural venous sinuses were observed at the site of the arachnoidal villi. An overcrowding of the spinal canal occurred so that the spinal cord was distorted and

the nerve roots herniated into the intervertebral foramina. These phenomena were believed to be strong evidence of the disproportionate growth ratio of the bone and the nervous tissue. In contrast, disturbances in growth produced by inanition or other specific nutritional deficiencies have been found to affect the rate of growth of skeletal and nervous tissues alike.

The repeated demonstrations by Wolbach and Bessey[10] under a variety of conditions strongly suggested that vitamin A has a specific effect on endochondral bone formation and that all nerve damage is secondary. Vitamin A is essential for the activities of the epiphyseal cartilage cells, without which they are incapable of carrying out the normal sequence of growth, maturation, and degeneration which is essential in the mechanism of endochondral or replacement bone growth. Since vitamin A deficiency suppresses epiphyseal cartilage cell sequences, endochondral bone growth is retarded and finally ceases entirely in long-continued A deficiency. Remodelling sequences, involving concurrent resorption of bone with bone deposition and replacement of cancellous bone by compact bone, cease to operate. There is a greatly reduced rate of resorption of trabecular bone with a retardation and failure of Haversian system formation which results in an arrestment of compact bone formation. All skeletal growth dependent upon replacement or endochondral bone formation ceases. Appositional growth of bone of periosteal origin continues until inanition intervenes at a rate in conformity to the normal growth pattern of each site. Presumably, the latter point indicates that there is no fundamental error of the calcification mechanism *per se* in the vitamin A-deficient animal.

Prenatal influences. A severe maternal deficiency of vitamin A has been found to result in the death and resorption of the fetus in pigs and rats. Degeneration occurs in the epithelial structures of the maternal organism and of the placenta.[12] When the deficiency state is less severe, the fetus is carried to term and has abnormalities which can be attributed to arrestment of growth in the skeleton and various organs. In pigs, where this effect was first reported, various stages of arrestment of formation of the eyes up to complete lack of eyeballs, harelip, cleft palate, misplaced kidneys, and extra earlike growths were observed.[13] In the offspring of vitamin A-deficient rats, Warkany and his co-workers observed an arrested development of eyes, pleural cavities, lungs, kidneys, testes, and diaphragm, as well as anomalies of the heart and blood vessels.[14-17]

A genetic trait which could be influenced by vitamin A deficiency has been described by Anderson.[18] In an inbred stock of albino rats, the incidence of congenital diaphragmatic hernia was 0.9 per cent when the vitamin A intake was normal. The incidence of this abnormality was increased to 18.9 per cent in the absence of a dietary source of vitamin A.

Hypervitaminosis A

General. The administration of excess vitamin A to experimental animals produces a complex and interesting syndrome. In the rat on a complete diet, hypervitaminosis A causes an increase in prothrombin time[19] and, in rats on a vitamin K-free diet, excess vitamin A will cause a high incidence of deaths by hemorrhage.[20] Vitamin A excess also causes a negative nitrogen balance, a

negative calcium balance, and a prompt marked negative phosphorus balance.[21] Excessive vitamin A administration causes healing in the metaphysis of the rachitic rat.[22] Epithelial structures which undergo keratinizing metaplasia in vitamin A deficiency are not influenced by the administration of excess vitamin A.

Bone, postnatal. That vitamin A has a potent effect on bone growth has been even more conclusively demonstrated by observations of the skeletal abnormalities which result from hypervitaminosis A in various species. The recently published studies in the dog, the duck, and the chicken are particularly good examples of the effect of this hypervitaminotic state.[23-25] When large amounts of the vitamin are administered to growing animals, the bones become extremely fragile, so that numerous fractures result. Comparable administration to adult rats does not result in any demonstrable effect on the skeletal structures over long periods. In this regard, the lack of influence in adult rats in comparison to the striking effect in growing rats is identical with the situation which exists between the adult and the growing rat in vitamin A deficiency.

Just as the normal sequences of epiphyseal bone growth are retarded in vitamin A deficiency, the same sequences are greatly accelerated in young animals by the ingestion of excess amounts of vitamin A. In all species which have been studied, there is a rapid maturation of epiphyseal cartilage cells and a more rapid penetration by the blood vessels. The rate of remodelling and the cartilage cell sequences are clearly related to the amount of vitamin A administered. Wolbach[26] interprets this action of excess vitamin A as an acceleration of "remodelling sequences in conformity to normal growth pattern. The remodelling takes place in spite of a retardation of linear growth of bone and is correlated with accelerated epiphyseal cartilage sequences. Those sequences retarded or suppressed in vitamin A deficiency are grotesquely and dramatically accelerated by the excess."

In the young guinea pig, rapid and complete consumption of the epiphyseal cartilage occurs. Osteoid production keeps pace with bone sequences resulting in a plate of bone and a greatly premature closing of the epiphysis. In the rat, closure of the epiphysis does not occur in hypervitaminosis A but this is not unexpected because closure does not occur in the normal rat.

Substitution of compact bone for cancellous bone in conformity to the normal growth pattern is greatly accelerated. Fractures and the sites of their occurrence are fully explained by acceleration of remodelling. Although new bone formation occurs as rapidly as bone resorption, maturation of the newly formed bone matrix and its calcification exhibit the same lag as in normal bone growth, and hence the bone at sites of remodelling is structurally weak. Bone which does not require remodelling in the normal growth pattern is not responsive to the administration of excessive amounts of vitamin A.

In the consideration of the graded responses of endochondral bone growth to vitamin A dosage through the entire spectrum from deficiency level to normal to excess, one cannot help wondering if this mechanism may not be under the mediation of some endocrine regulation. At present, there is little data on which to evaluate such a premise. The one piece of information of this type concerns hypervitaminosis A in hypophysectomized rats. The hypophysectomized and

the normal rat respond in similar fashion to excess vitamin A injection. When the retardation of bone growth resulting from hypophysectomy in growing rats is taken into consideration, no differences in responses to excessive vitamin A were noted as compared to normal rats.[27] Even earlier fractures were noted in the hypophysectomized rat. This response was thought to be attributable to the slower rate of new bone formation expected in hypophysectomized rats by reason of the over-all slower growth pattern. Thus, the hypophysis is not required for mediation of the skeletal growth-promoting influence of vitamin A. There is a real need to examine the role of other endocrine glands with respect to this effect.

Teeth. The formation of dentin and enamel may be accelerated in periods of vitamin A excess, but there is no apparent departure from the normal structure. This response is not unexpected, since no remodelling sequences are involved. Since adequate studies of the influence of hypervitaminosis A on tooth growth have not been made, there may be effects on rate and type of dentin and enamel formation which have not been characterized. The bone supporting the teeth exhibits accelerated growth sequences comparable to those elsewhere in the body.

Metabolic Function of Vitamin A

Despite the detailed morphological knowledge about the response of the calcified tissues to vitamin A deficiency, apparently there is still no information available on the histochemical or biochemical level to suggest what failure at cellular levels precipitates the degeneration of cells of epithelial origin, including the ameloblasts, or the cessation of the normal epiphyseal-cartilage cell sequences. Our knowledge of the general physiologic role of vitamin A in the body does nothing to channel our thinking into any specific path for an initial exploration in those tissues. Indeed, the only specific function of vitamin A which has been elaborated in biochemical terms is its essential participation in the visual system process. The visual biochemistry of vitamin A has been recently reviewed by Wald.[28] Unquestionably, this phenomenon, though a dramatic one, is of relatively minor importance in the totality of vitamin A metabolism in so far as it participates sufficiently intimately to be necessary for the very life of the animal as well as its skeletal growth.

Ascorbic Acid Deficiency

General. Only primates and guinea pigs are known to require an external source of ascorbic acid. The deficiency disease attributable to this vitamin is known as scurvy. The outstanding signs are hemorrhages in widespread areas of the body, especially in intramuscular and subcutaneous areas, and a general weakness of the tissues, especially in areas where there is a relatively high content of collagen and related substances. There is a general agreement among investigators that there is a partial or complete inability to form intercellular substances throughout the entire body. Intercellular substances such as collagen, osteoid, and predentin fail to be deposited by their respective cells, the fibroblasts, osteoblasts, and odontoblasts. The entire pathologic picture of

scurvy apparently can be explained on this basis, since the various morphologic changes appear to be consistent with structures or processes which are dependent upon the elaboration of intercellular substances.

Teeth. In 1919, Zilva and Wells[29] reported studies which indicated that the teeth were probably the first organs in the guinea pig to be affected by the deficiency of ascorbic acid. Then Höjer[30] suggested that the microscopic changes in the teeth of the guinea pig were sufficiently closely related to the amount of this vitamin in the diet to permit the development of an assay method.

Wolbach and Howe[31, 32] and Boyle, Wolbach, and Bessey[33] studied the pathogenesis of the changes extensively. Since the odontoblasts are of entodermal origin and, in normalcy, elaborate an organic matrix containing typical collagenous intercellular substances, the most marked alterations in the teeth during scurvy are found in the odontoblasts and the formation of dentin. When guinea pigs are maintained on a scorbutic diet, alterations very soon appear in the odontoblasts. These cells become atrophic and soon resemble the nearby pulp cells. There is a decrease in their orderly polar arrangement, a decrease in height and, eventually, a complete disorganization. The vessels of the pulp become dilated and red blood cells ooze through. As a result of the changes in the odontoblasts, dentin is laid down irregularly and at a greatly reduced rate, and the dentinal tubules are arranged in haphazard fashion. The rate of dentin formation is sufficiently closely related to vitamin C intake that it has been suggested as a criterion upon which to base a bioassay for vitamin C content.[34] The decrease in height of the odontoblasts in moderate deficiencies is believed to be sufficiently closely related to the vitamin C intake to permit this measurement to be used as a bioassay criterion.[35]

In severe deficiencies, dentin deposition soon stops entirely and the predentin becomes hypercalcified. A few of the odontoblasts in the pulp apparently are capable of forming some dentin, at least enough to enclose themselves. In the guinea pig, changes in the enamel organ come later in the course of the deficiency.[36] The ameloblasts atrophy and hemorrhages are encountered. Both these alterations are interpreted to be due to traumatic injury of the enamel organ as a result of inadequate support by adjoining tissues. There is a rarefaction of the alveolar bone, as might be expected, when one recalls the changes encountered in the ribs and other bones of the experimental animals and humans. This pathologic sequence in destruction of the alveolar bone has been reported to resemble closely the changes observed in diffuse alveolar atrophy.[37] Weakness of the supporting bones, as well as weakness of the collagen fiber-supporting apparatus, allows for greater mobility and decreased ability to withstand the mechanical stresses encountered in chewing.

Westin[38, 39] stated that the pathology in the pulp and odontoblastic layer of the teeth in human beings was nearly identical with the pathologic changes in the scorbutic guinea pig. In the teeth of scorbutic adults, the dentin was resorbed and porotic. The small amount of replacement dentin was of the osteodentin type. The pulp was found to be atrophic and hyperemic. Degeneration of the odontoblasts, the formation of cysts and foci of denticulelike regions of calcification were observed.

Boyle[40] examined the teeth of two scorbutic infants, but was unable to detect any changes comparable to those in the formative dental tissues of the guinea pig.

The relationship of ascorbic acid deficiency to gingival changes has been repeatedly demonstrated. Descriptions of these changes and their clinical implications are beyond the scope of this review and have been presented in detail elsewhere.[41] However, there has been no clearcut example of a relationship of scurvy to dental caries. A large experiment by Hanke[42] at an orphanage has been interpreted to indicate that the supplementation of the orphanage diet with a pint of orange juice and the juice of one lemon daily over a period of one year materially reduced the incidence of new carious lesions. Surveys by Hess and Abramson[43] and experiments by McBeath[44] and by Grandison, Stott, and Cruickshank[45] failed to demonstrate any difference in dental caries incidence between the children in the control and experimental groups.

Bone. In early stages of vitamin C deficiency, the cartilage cells of the epiphyseal plate continue to proliferate and arrange themselves in normal rows. Likewise, mineral salts are deposited in the cartilaginous matrix substance between the columns of cartilage cells. However, the osteoblasts fail to lay down osteoid on the spicules of calcified matrix material which, in addition, is not destroyed. Thus, a wide zone of calcified, but unossified matrix develops just beneath the actively growing cartilaginous plate. Since this matrix, actually, is a "lattice" of calcified catilaginous matrix material, Park[46] has given it the designation of "scorbutic lattice." The development of this zone determines the final pathologic picture, because such spicules of calcified matrix, without normal bone surrounding the spicules, have a low resistance to the ordinary stresses and strains and, hence, are especially liable to fracture.

The first site of the appearance of fractures is usually where the cortex and the cartilage are in juxtaposition. As the lattice increases in width to form a more and more fragile zone, it becomes inevitable that complete fracture of the spicules of lattice will occur, and that separation will occur at the cartilage shaft junction with various accompanying deformities. Such fractures of the calcified matrix material result in the classical textbook picture of scurvy, in which there is a region of complete disintegration, with spicules of calcified matrix in considerable disarray, lying horizontally and in various other directions beneath this cartilage.[47]

When one hind leg of a guinea pig is immobilized by placing it in a plaster cast, and the animal is then placed on a scorbutic diet, interesting differences between the hind legs are observed.[48] At the cartilage shaft junction of the immobilized tibia, there is a broad zone of calcified lattice, but no evidence of fractures, hemorrhage, hyalin, pink-staining material, nor proliferation of fibroblastic or osteoblasticlike cells. In contrast, the tibia of the opposite side exhibits the classical picture of scurvy with all of the positive findings. Such an experiment shows that, with the exception of a prominent lattice of calcified matrix material, all of the above descriptive criteria of scurvy are secondary to the effects of mechanical force on this area of structurally lower resistance.

Repair in scurvy begins within 24 hours and is evident both in the resumption of growth of cartilage cells and in matrix deposition.[49, 50] Within 48 hours,

sufficient matrix is laid down to produce the effect of capsules, and enlarged, maturing cartilage cells have become finely vacuolated. In 72 hours, cartilage cells are visible which appear to be fully matured, and there is a restoration of the columnar arrangement. By 96 hours, appearances approach normal, and some penetration of diaphyseal blood vessels may have occurred. In addition, osteoid is deposited in normal distribution and there is resumption of appositional bone formation.

The known relationships of cortisone in the clinical treatment of diseases which are assumed to have their origin in collagenous structures prompted the investigation of whether cortisone would have any effect upon the cessation of formation of the intercellular substances of fibrous tissues, bone, and cartilage in scorbutus and on the resumption of formation of these materials, during recovery. Wolbach and Maddock[50] reported that the administration of cortisone prophylactically or therapeutically, after the development of scurvy, does not modify the gross or histologic appearance of the events in the progression of the scorbutic state, and does not alter the rate at which intercellular substances form during recovery from scurvy. These observations would suggest that hormonal control of this process through this pathway is unlikely.

Metabolic Function of Ascorbic Acid

Just as in the case of vitamin A, little or no analytical data exist to suggest the mechanism whereby vitamin C and the cells requiring vitamin C elaborate their products. Unquestionably, through some mechanism, ascorbic acid regulates the colloidal condition of intercellular substances which contain collagen or related substances.

Höjer[39] was probably the first of numerous investigators to propose that the antiscorbutic substance acted directly on the protoplasm of the highly differentiated mesenchymal cells, fibroblasts, osteoblasts, and odontoblasts, which are the most sensitive to vitamin C deficiency. These cells, then, were supposed to be capable of the elaboration of properly oriented intercellular substances. Support for this site of action has been furnished by electron microscope studies on tissue culture material.[51] Most of the ectoplasm layer of the cell appears to be involved in fiber production, and the collagen seems to differentiate out of the surface as bundles of fibrils which increase in diameter approximately threefold after separation from the cell.

Proposals have been made that ascorbic acid exerts its influence directly on the extracellular material. Wolbach and Howe,[32] in particular, were proponents of this hypothesis and have presented strongly suggestive experimental evidence. The rapidity with which formation of intercellular substances occurs during repair is considered by these investigators to be evidence that the cells do not participate actively in the process at this stage.

The mode of action of ascorbic acid, it has been suggested, may resemble that of a catalyst or of a component of the compounds of structural or enzymatic nature. At present, there is no definite evidence that vitamin C or any derivative is used as a structural acid in any compound in cells or in intercellular substances. Some evidence is available to indicate that ascorbic acid may function as a coenzyme in the oxidation of tyrosine.[52, 53] Anent the question of a catalytic

activity for ascorbic acid, a number of proposals have been advanced with relatively little substantiating evidence. An effect on surface activity of cells has been proposed by reason of certain demonstrated influences on the surface activity of colloidal solutions.[54] Indistinct blurred cellular outlines have been reported in the embryo of scorbutic animals.[55] Markedly reduced phagocytic activity and increased fragility of polymorphonuclear cells in scurvy is another suggestion of altered surface activity.[56]

Vitamin D Deficiency

General. The primary influence of vitamin D appears to be on the absorption of calcium from the intestine with a secondary influence on the absorption of phosphate.[57] Rickets is the developmental disease of the calcified structures that results from vitamin D deficiency, from calcium or phosphorus deficiency, or from combined deficiencies of these nutrients. Osteomalacia is the comparable adult disease attributable to the same nutritional deficiencies or to factors which precipitate these deficiencies. The various causes of rickets and osteomalacia have been dealt with in detail by Albright and Reifenstein.[58]

The experimental production of rickets in the dog was accomplished by Sir Edward Mellanby, through the use of a diet which was deficient in other nutrients, including vitamin A.[59, 60] Mellanby observed that this vitamin exerted a specific controlling influence over many of the functions of growth in the body and, in particular, over the development and calcification of bone. His wife noted that a deficiency of this fat-soluble vitamin in young puppies had a profound effect on the developing enamel of the permanent teeth, on the rate of eruption, and on the position of the teeth in the jaw.[61]

Rickets; bone. The morphological criteria of rickets are found in the bony shaft and at the junction of the shaft and the cartilage. Two separate processes are involved: first, the failure of epiphyseal cartilage cells to complete the sequences of proliferation, maturation, and degeneration; and, second, the failure of osteoid matrices to calcify. Apparently, the changes are closely similar whether produced by vitamin D deficiency in species which require it, or by calcium or phosphorus deficiency, or by imbalanced calcium-phosphorus ratio. One possible exception is in the rat, where hypertrophic, uncalcified cartilage and osteoid appear to be much more prominent in diets with high calcium-phosphorus ratio than in those with low calcium-phosphorus ratio. The degree of change which will be encountered at the cartilage shaft junction is dependent on the rate of growth of the bone which is dependent, to a large extent, on the age of the organism.

In the normal growth of the trabeculae and cortex of the shaft, constant remodelling sequences are being undertaken. The trabeculae are in a continual state of flux, being removed and replaced as the requirements of the body dictate. The inner margin of the cortex, particularly when growth in diameter is taking place, is removed, and appositional growth occurs along the external surface. In rickets and in osteomalacia, osteoblastic activity is not affected, except when inanition or some intercurrent disease intervenes. Osteoid, the organic matrix of bone, is deposited upon pre-existing bony trabeculae in normal fashion. However, inorganic salts are not deposited in this osteoid. Whereas

the deposition of inorganic salts occurs, to all intents and purposes, almost simultaneously with the deposition of osteoid in normal animals, in rickets, the deposition of lime salts is either retarded or completely lacking. The amount of normal or physiological osteoid varies, depending upon the age and the species from which the specimen is derived. In the normal growing rat, osteoid is rarely detectable. Follis states that "Osteoid is not ordinarily encountered in older children (after two years) and in adults; if present in these, it denotes rickets or osteomalacia. In rickets and osteomalacia osteoid borders of uniform thickness do not cover each and every trabecula; on the contrary, the deposition of osteoid is usually irregular and is doubtless related to mechanical stress and strains. The absence of osteoid does not connote that rickets is not present; changes at the cartilage shaft junction may be recognized in certain instances in which osteoblastic activity in the shaft is so reduced, as by wasting disease for instance, that little or no osteoid is deposited."[47]

At the junction of the cartilage and shaft, the cartilage cells reach their maximum size, produce adequate amounts of matrix, but fail to degenerate. As proliferation and increase in cell size occur, the epiphyseal cartilage increases in width. By reason of the lack of degeneration of cartilage cells, there is no space created for the ingrowth of cartilage. If capillaries do invade the cartilage, it is in a very irregular fashion. At the same time, the matrix fails to calcify in the neighborhood of the cells which ordinarily would have begun to degenerate.

The accumulated cartilage and osteoid mass frequently becomes misshapen by the pressure of weight bearing and often results in stratification of the cartilage. Where the rachitic process is long standing, there is a disappearance of the trabeculae and resorption of cortical bone to supply more demanding needs of the body for calcium.

When therapy is begun, repair starts to occur rapidly. The first visible evidence is the resumption of cytomorphosis within 24 hours, as seen by the presence of cleared or degenerated cells on the diaphyseal border of the cartilage. Within 48 hours, cell maturation is accompanied by extensive penetration of capillaries and concurrent calcification of adjacent matrix.[9] Inorganic salts are deposited in various parts of the rachitic junction of the cartilage and shaft. Calcification occurs first in proximity to the capillaries which have invaded the spaces left by degenerated cartilage cells. The deposit proceeds toward the diaphysis until there is a calcification of the accumulated osteoid which apparently cannot be removed prior to calcification. The entire area is ultimately remodelled. Where there are no deformities of the bones, the remodelling process results in structures which grossly and histologically are normal. Where deformities exist, as in the bending of the long bones, the remodelling processes are incapable of correcting the abnormalities in shape, although the histological changes are corrected. Detailed X-ray diffraction studies of rats, after the rachitic process appeared to be healed by routine tests, showed abnormal crystal patterns which persisted for prolonged periods and which, in some cases, were not completely eradicated.[62]

Vitamin D has an interesting relation to the citrate content of the bones. In 1941, Thunberg reported that the bone substance of the horse, ox, pig, sheep,

hen, and five species of fish contained as much as one to a few per cent of citrate.[63] He also reported that about 0.5 per cent was found in the teeth of the calf, ox, and pig. The same year, Dickens reported that bone from a rachitic cat contained about half as much citrate as bone from a comparable normal animal.[64] In contrast, a puppy which had received prolonged administration of parathyroid hormone had approximately 50 per cent higher level of citrate in the bone than a normal puppy of the same age. A rather striking species difference was also noted. Nicolaysen and Nordbø added further information to Dickens' observations when they noted, in vitamin D deficient rats, that the reduction in citrate was greater than the reduction in ash content of the bones.[65] This finding was in direct contrast to the effect of mineral starvation, wherein a strictly stoichiometric reduction in ash and citrate was observed. Waasjö and Eeg-Larsen noted that additions of phosphates to a high calcium-low phosphorus rachitogenic diet resulted in an increase in the ash content, but with no increase in citrate.[66] Furthermore, the ingestion of citrate at a level of 5 per cent in the diet had no influence on the bone citrate level or on the ash content in rachitic rats. When vitamin D was given to rachitic rats, they noted that the citrate content of the bones returned to normal within the first five to ten days, whereas the ash content responded much more slowly. Eeg-Larsen has noted a difference in concentration of citrate which is interesting.[67] The level in the diaphysis was about 0.7 per cent, whereas in the epiphysis there was only 0.4 per cent. These observations were made in the rat and are in contrast to Cartier's studies in 1948, where a comparable distribution was noted in various areas of rabbit long bones.[68] No comment was made about the relative age of the two species, so that the growth pattern may well have influenced the citrate distribution. Eeg-Larsen also noted that the lactate content of the tibias of young rats was almost twice as high in the epiphysis (0.4 per cent) as in the diathesis (0.2 per cent). Glycogen was largely localized in the epiphyseal carilage at a level of about 0.7 per cent of the dry weight. In rickets, the glycogen and the lactate content of the epiphysis was significantly higher than in the controls. Glycogen and lactate vary inversely with the citrate content, both with respect to localization in the long bones and also with respect to the influence of vitamin D on these metabolites. In earlier studies, Hamilton and Dewar[69] and Hathaway and Meyer[70] reported that the ingestion of citric acid and citrates at levels of 10 to 15 per cent had a specific calcifying effect when added to a rachitogenic diet. However, it is noteworthy from the studies of Nicolaysen and Nordbø that the injection of large amounts of citrate had no effect on the calcifying process. It has been proposed that the citrate or citric acid in the diet promotes the formation with the calcium in the intestines of a complex which is more readily absorbed than the calcium ions.

Rickets; teeth. The changes which occur in the teeth in rickets are less complex than those in bones, mainly because no portion of the former are ever remodelled. When young rats are placed on a rachitogenic diet (high calcium, low phosphorus, no vitamin D), the first and most prominent change is in the incisors, where a line of disturbed calcification appears in the dentin—the "calcio traumatic line."[71] This, actually, is the first histologically detectable response of the organism to the effects of the rachitogenic regimen. It is accompanied

by a retardation in the formation of predentin and a pronounced disturbance in the calcification of all forming dentin which is no longer homogeneously basophilic but is stippled by an irregular deposition of inorganic salts. Calcification of the cementum is likewise retarded. The changes in the developing molars are similar but less severe.

Although there are cystic alterations in the enamel organ before it undergoes atrophy, no other abnormalities in this structure were detected. No enamel hypoplasia has been observed in rats although, in the guinea pig and the dog, severe hypoplasia of the enamel has been reported, when these animals are placed on a low calcium-high phosphorus diet containing no vitamin D.[61, 72] In experimental animals, specific differences may exist, although the calcium and phosphorus concentrations of the diet were reversed, which may explain the presence or absence of enamel hypoplasia. Furthermore, Mellanby[73] has observed that in dogs the abnormalities in enamel and dentin were greater in calcium deficiency with ample vitamin D than in a simple vitamin D deficiency.

Actually, no categorical statement can be made about the influences of various calcium-phosphorus ratios, as well as various absolute amounts of calcium and phosphorus, with and without adequate vitamin D, on the histological appearance of the developing enamel and dentin. There is a real need for intensive investigation in this area.

The bony supporting structures of the teeth show characteristic changes similar to those of the bones described in preceding paragraphs. Wide osteoid borders are found on the trabeculae of the alveolar bone. The number and size of the trabeculae are greatly decreased.

Enamel hypoplasia in man is, without doubt, frequently the result of a rachitic process during tooth development, although this is by no means to be considered the only etiologic factor.[74, 75] The question of whether enamel hypoplasia of gross or microscopic degree is related to the caries-susceptibility of teeth has often been debated with inconclusive results. Mellanby[76] has been one of the main proponents of a positive correlation between tooth structure and dental caries incidence. In 1500 sectioned deciduous teeth, 78 per cent of these with well calcified enamel and dentin were free from caries, while only 6 per cent of the very hypoplastic teeth were caries-free. Extensive dental caries was observed in only 7.5 per cent of the former teeth, in contrast to 74 per cent of the latter group. A similar association was observed in 275 sectioned permanent teeth.

Various studies by Mellanby and her co-workers have indicated that an adequate vitamin D intake in growing children was responsible for a reduced incidence of dental caries.[77, 78] No claims were made that adequate vitamin D nutriture would completely prevent dental caries. Several comparable studies have been conducted by several investigators with essentially the same positive results.[79]

Prenatal influences. Warkany[80] has described the abnormalities which occur in the offspring of rats fed a diet lacking in vitamin D and having a high calcium-low phosphorus content. The female rats produce offspring 45 per cent of which had congenital skeletal abnormalities. Pronounced curving of the radius, ulna, tibia, and fibula, as well as angulation of the ribs was reported.

The histologic picture did not appear to be characteristic of rickets, since there was no excess osteoid. However, the same diet produced typical rachitic changes in young rats. From this result, it would appear that foetal tissues reacted somewhat differently to the deficiency or, possibly, the lack of stress on the foetal tissues may have been responsible for the different appearance of the deficiency.

A profound effect of vitamin D deficiency during pregnancy and lactation upon the deciduous teeth of the dog has been described by Mellanby.[81] When the diet of the female dog during pregnancy was deficient in vitamin D, the deciduous teeth had defects in structure and calcification, and their eruption was delayed.

Hypervitaminosis D

The ingestion of excessive amounts of vitamin D results in pathologic changes in bones and soft tissues. The initial administration of a massive dose of vitamin D results in a great increase in serum calcium which appears to be the effect of an enhancement of the normal physiologic influence of vitamin D on calcium absorption from the gut. The degree of calcium absorption and of blood calcium increase appears to be stoichiometrically related to the amount of vitamin D ingested.

The most pronounced features of prolonged vitamin D toxicity are the dissolution of the bone shafts by resorption of cortical bone with noncalcified matrix deposits of periosteal and endosteal origin and the formation of short bones with noncalcified trabeculae of unusual thickness and number at the epiphyseal ends.[3] The most dominant toxic effect in the soft tissues is the precipitation of calcium phosphate in the kidney tubules. Comparable inorganic salt precipitation occurs in a variety of other soft tissues. There appears to be no disagreement in the literature to the hypothesis of Guldager that damage in the soft tissues is never seen before the precipitation of salts.[82] The time of appearance of the signs of hypervitaminosis D, the severity of the symptoms, and the survival depend upon the amount of calcium absorbed, the degree of bone dissolution, and the ability of the kidney to excrete. When a single massive dose of vitamin D is given, the deposition of salts occurs concurrently with the secondary fall of serum calcium, not with the initial increase nor during the period of high serum calcium.[83]

Metabolic Function of Vitamin D

With respect to the physiology of vitamin D, our knowledge is far from complete. In species such as man and the dog, there has been a long standing demonstration that vitamin D is undoubtedly the most dominant, single factor in the regulation of the absorption of calcium, which subject has been reviewed in detail recently.[57, 84] In addition, there is an influence on the phosphorus absorption which is secondary to and largely determined by calcium absorption.[57] Vitamin D deficiency in these species results in reduced blood calcium and phosphorus, reduced ash content of the bones, increased phosphatase activity in the bone cartilage and osteoid tissue, and an increased phosphatase level in

the blood, and also increased levels of calcium and phosphate in the feces. In the dog and man, fecal elimination approximates intake in acute rickets. In contrast, the intact rat in advanced rickets still is capable of absorbing appreciable amounts of calcium and phosphorus. However, it is noteworthy that the rat absorbs more calcium and phosphorus in the presence of vitamin D than in its absence.[86] Since the rat does not require vitamin D for the absorption of calcium, pure vitamin D deficiency can be produced and studied in this species uncomplicated by a contributory calcium deficiency.

The physiological relationship of vitamin D to the absorption of calcium and phosphorus is inadequate to explain a number of observations in man and experimental animals. The intravenous administration of calcium and/or phosphorus salts results in increased blood levels in species which require vitamin D for calcium absorption.[56, 57] In the presence of vitamin D, healing can begin without food ingestion or during the ingestion of a calcium-phosphorus free diet.[88] Administration of parathyroid hormone results in increases in blood calcium, but with little or no antirachitic effect in species which require vitamin D for calcium absorption.[89] In addition, Nicolaysen and Jansen[90] have published observations which indicate that vitamin D influences the structure of the bone matrix in the rat independently of the calcium and phosphorus absorption effects. These studies are all reminiscent of the early *in vitro* studies in which calcification occurred in the presence of serum from normal animals or from animals with healing rickets, but did not occur when the serum was from a rachitic animal.[91, 92] Altogether, these facts point to an action of vitamin D on an intimate cellular level to promote normal cellular development and calcification of the matrix.

A third primary role of vitamin D is in relation to the citric acid content of bones, as described in a preceding section.

Harrison and Harrison[59] proposed that vitamin D had a specific effect on kidney tubular reabsorption of phosphate. This seems unlikely in view of the fact that no phosphorus diuresis in vitamin D deficiency has been recorded. Albright and Reifenstein[75] interpret the evidence for reabsorption of phosphorus presented by Harrison and Harrison to be indicative of a decreased parathyroid activity prompted by the increased blood calcium level, due to the administration of vitamin D in their experiment.

A possible enzymatic function for vitamin D has been suggested by Zetterström,[93, 94] who studied the effect of phosphorylated vitamin D upon the action of alkaline phosphatases from kidneys, bones, and intestines. The rate of the enzymatic release of the phosphate from phenyl phosphate was approximately doubled in the presence of the phosphorylated vitamin. Vitamin D also appears to activate phosphatase activity toward glucose-6-phosphate.

It would seem that all these influences together still did not account for the total effect of vitamin D in the animal body. Further exploration of the role of vitamin D is necessary before the final analysis of its function can be written.

Pantothenic Acid Deficiency

During the period when the filtrate fraction of the B-complex was being studied to determine its importance and its chemical nature, Becks and Morgan[95]

reported that a deficiency of this fraction, which later was recognized to contain pantothenic acid, caused an advanced osteoporosis in the mandibular bone of the dog with extensive resorption of the molar roots. Severe odontoclastic activity was reported deep in the dentin, calculus formation was pronounced, and there was an indication of a high incidence of tooth decay. This condition was accompanied by considerable periapical inflammation with formation of granulation tissue.

Later Levy and Silberberg[96] reported that pantothenic acid deficiency in growing mice caused an inhibition of skeletal growth and endochondral ossification. The epiphyseal disc was only half as wide as in the control, the cartilage cell rows were regular, but they contained only from 4 to 6 inactive columnar and from 1 to 3 hypertrophic cartilage cells instead of the usual number of 10 columnar and 4 hypertrophic cells. Ground substance was more abundant than in the controls, and calcification of provisional cartilage proceeded at a regular rate. The metaphysis contained few and collapsed capillaries. The trabeculae were much shorter and scarcer than in the controls and were covered with a discontinuous layer of small, flat spindle cells, instead of the usual osteoblastic cell layer. In the adult mouse, where growth had ceased, no skeletal abnormalities were observed despite severe pantothenic acid deficiency.

Later Levy[97] described effects of pantothenic acid deficiency on the mandibular joint of mice. These changes were similar in character to those in the knee joint. There was an inhibition of proliferation and of hypertrophic growth of the mandibular condyle resulting in a mandible of less than normal size. Maxillary growth was not retarded as greatly as growth in the mandible, so that a malrelation of the jaws was produced. The over-all oral changes were characterized by the definite lack of bone formation, continuing resorption of the alveolar bone, narrow interdental septum, a broad periodental membrane with a low alveolar crest.

Nelson and co-workers[98] have reported a series of abnormalities in endochondral ossification during pantothenic acid deficiency in young rats. Four different stages of changes were observed. In stage one, when the rats were between 21 and 33 days of age, it was characterized by unusual trabecular resorption, decreased osteoblast proliferation and edema of the bone marrow. In stage two, from 33 to 63 days, there was a marked retardation of osteogenesis with some calcification in the epiphysis and blunt diaphyseal trabeculae. The dominant feature in stage three, from 65 to 72 days, was the beginning of epiphyseal cartilage calcification and the cessation of osteogenesis. Stage four, 78 to 100 days, was characterized by complete absence of trabeculae and the formation of a heavy layer of sealing-off bone below the epiphyseal cartilage.

Frandsen and others[99] have also studied the growth and transformation of the mandibular joint, this time during pantothenic acid deficiency in the infant rat. Feeding of the pantothenic acid deficient diet began at birth. The changes in the condyle were characterized by marked impairment of chondrogenesis and osteogenesis and, in the late stages, by necrosis of the articular capsule, the articular disc, and the fibrous tissue in the glenoid fossae resulting in an almost complete destruction of the mandibular joint. The severity of the changes depended more or less on the individual response rather than upon the

duration of the deficiency. Fibrous tissue replaced the bone marrow and the disintegrated bone trabeculae. The inhibition of chondrogenesis and osteogenesis was much more severe than had been reported by Levy[97] for pantothenic acid deficiency in the mouse. This difference is not unexpected, in view of the fact that the acute syndrome described by these workers resulted from the imposition of the deficiency from birth rather than from the time of weaning.

Lefebvres-Boisselot[100] has described the occurrence of a substantial incidence of gross malformation in rat embryos. The most conspicuous abnormalities were hydrocephalus, anophthalmia or microphthalmia, exencephaly, and edema.

No studies of the effect of pantothenic acid deficiency on the development of the teeth are known to have been reported.

Choline Deficiency

Wolbach and Hegsted[101] have studied the effect of choline deficiency on the epiphyseal cartilage of the chick. The gross syndrome produced by choline deficiency has been termed "perosis" or "slipped tendon."[102] The most conspicuous sign of this deficiency is an enlargement, deformity, and disability of the hock joint: *i.e.*, at the articulation of the tibiotarsus and tarsometatarsus. The primary pathological change in this syndrome is in the epiphyseal cartilage. The early changes in epiphyseal cartilage sequences were in the zone of enlarging cells and in the zone of maturing cells. After 14 days on experiment, all epiphyseal cartilage zones were involved in the choline deficient chicks. The changes could be characterized as a failure of cartilage cells to mature, the presence of an excess atypical matrix in the zone of growth, and an excess of matrix with a reduction of mitoses in the zone of proliferation. Tunneling of the cartilage is retarded or completely suppressed. There is no apparent defect in osteogenesis, therefore all of the mature cartilage cells of tunnel walls become replaced by bone, with the result that there is an almost complete disappearance of the cartilage columns characteristic of normal endochondral bone growth. The epiphyseal-diaphyseal junction becomes changed from a lengthy structure of cartilage tunnels lined with bone to a narrow zone where penetration of bone has been abruptly prevented by failure of maturation of the cartilage cells.

These investigators believe that the result is a weak union of the epiphyseal cartilage and the diaphysis with the resultant bowing of the ends of the bones of the hock joint. No evidence that bone deposition, both appositional or wherever epiphyseal cartilage sequences are permitted, was abnormal. There was no evidence that the changes in the epiphyseal cartilage contribute directly to the deformities of the hock joint, although the presence of an increased amount of atypical matrix suggests participation of the cartilage. In the zone of proliferation, which is normally composed of flat cells, transversely arranged to the long axis of the bone, there is a loss of the sharp line of demarcation from the zone of growing cells because of an increase in the size of the cells and in the matrix. This line of demarcation between the epiphyseal cartilages of femur and tibia and articular cartilage may be irregular, owing to enlarged epiphyseal cartilage cells surrounded by matrix. These changes became more pronounced as the duration of the deficiency was increased.

Comparable studies with rats have not been reported, and no studies of the effect of choline deficiency on tooth development are known to have been conducted.

Riboflavin Deficiency

No suggestions of bone or tooth abnormalities have been reported postnatally for riboflavin deficiency. However, Warkany and his co-workers[103] described a series of abnormalities in the offspring of female rats which received a riboflavin-deficient diet. A high proportion of the offspring displayed a shortening of the mandible, radius, ulna, tibia, and also syndactylism, cleft palate, a thin abdominal wall in the ventral midline, and protrusion of the abdominal viscera. When hens are fed a riboflavin-deficient diet, almost all the chicks which hatch from their eggs have micromelia.[104]

Folic Acid and Vitamin B_{12} Deficiencies

Richardson and Hogan[105] reported the occurrence of hydrocephalus in 2 per cent of the offspring from female rats on a synthetic diet. Later, they demonstrated that this malformation was partially prevented by the addition of pteroylglutamic acid to the maternal diet.[106] The addition of sulfa drugs to the diet[107] or the incorporation of a folic acid antagonist[108, 109] increased the incidence and severity of the syndrome. Folic acid deficiency in the diet of the laying hen causes a high incidence of syndactyly, deformed jaws, and parrot beak in the embryos.[110]

In other studies, Hogan and his co-workers showed that the incidence of hydrocephalus in infant rats was increased to 28 per cent when the maternal diet was deficient in vitamin B_{12}. There was a smaller incidence of spina bifida, small or missing eyes, cleft palate, shortened lower jaw, and short or kinked tails. The addition of a vitamin B_{12} concentrate to the diet or the injection of crystalline vitamin B_{12} in the early stages of pregnancy prevented the hydrocephalic syndrome in the young and increased their viability.[111] Vitamin B_{12} deficiency has also been shown to cause perosis, myoatrophy, and hemorrhage in chick embryos.[112]

Neither of these nutrients has been reported to cause bone or tooth abnormalities during postnatal life.

Biotin Deficiency

Couch *et al.*[113] have described a series of abnormalities in the embryos from eggs laid by biotin-deficient hens including perosis, shortened and bent tibiotarsus, shortened tarsometatarsus, shortening of the bones of the wing and of the skull (parrot beak), and a shortening and bending vertically of the anterior end of the scapula. Jukes and Bird[114] have reported that there was an incidence of 55 per cent perosis in biotin-deficient chicks. No histopathologic studies are known to have been conducted of the perosis syndrome attributable to biotin deficiency.

Vitamin E Deficiency

Dam, Granados, and Maltensen[115] have reported a series of studies on the effect of vitamin E deficiency on the incisor of the rat. The enamel of the

portion of the incisor which developed during vitamin E deficiency was depigmented. No appreciable variation in calcium, phosphorus, and magnesium concentrations of the enamel and dentin of the incisor was observed between experimental and control rats. Iron was decreased dramatically in the enamel of the vitamin E-deficient incisor. An unusual situation was observed with respect to the manganese content of the enamel and dentin where substantial increases were noticed in the incisors from vitamin E-deficient rats.

Potassium Deficiency

Potassium is not commonly thought to be concerned with calcification in any intimate fashion. However, Gillis[116] has reported studies in chicks and rats which indicated substantially lower concentrations of bone ash in potassium-deficient subjects. A significant reduction in the inorganic phosphorus concentration of the blood plasma was observed in potassium-deficient animals. No alteration in blood calcium was observed. The amount of potassium required for optimum calcification was less in the presence of an adequate intake of phosphorus than when phosphorus was supplied at an inadequate level.

Magnesium Deficiency

An early manifestation of magnesium deficiency in the rat is the retardation of dentin formation.[117-121] This retardation was particularly true for the dentin on the labial surface, which is half or less the width of the lingual dentin. Peculiar striations in the dentin were evident which were thought to be due to variations in growth similar to those which are seen in the bones. The odontoblasts are responsible for the changes in the dentin since these cells become atrophic and are enclosed on all sides by dentin. In a similar fashion, the ameloblasts atrophy. Hence enamel formation is retarded and the resultant enamel is hypoplastic. Calcified stones are also a prominent feature in the pulp of magnesium-depleted teeth. Chemical studies have shown no great decrease in the absolute magnesium content of the rat's incisor formed during magnesium deficiency,[122] unlike the situation in bone, where magnesium has been shown to decrease and calcium to increase in the early stages of the deficiency.[123] In this respect, magnesium may fall into the same category as sodium which, as Nichols and Nichols have shown, is able to leave the bones without simultaneous destruction of the latter.[124]

Histological studies of the growing bones of magnesium-deficient animals apparently have not been reported.

Manganese Deficiency

Changes in the bones of manganese-deficient animals have been noted by several observers. Barnes *et al.*[125] presented roentgenographic evidence that the tibiae of manganese-deficient rats were shorter than normal, and that the epiphysis of the proximal end of the tibia was narrower than normal. Shils and McCollum[126] have noted a shortening and bowing of the forelegs in the same species and in the mouse. Amdur, Norris, and Heuser[127] observed that the deficiency of manganese in the rat caused a decrease in the length and density, as well as in the breaking strength, of the bone. Phosphatase activity is

also reduced. In accordance with the observations of others, no differences have been found in the percentage of ash or of calcium, phosphorus, and manganese contents.

Wolbach and Hegsted[101] have studied the effect of manganese deficiency on the epiphyseal cartilage in the chick. The syndrome produced by this deficiency is grossly similar to that produced in choline deficiency. Upon examination of the pathological changes in manganese deficiency, these investigators were unable to detect any differences in the cellular changes which occurred from those that occurred in choline deficiency. They commented upon the fact that, in choline deficiency, the changes seemed to occur more quickly and to a greater degree. However, they noted that the rate of growth was much more stringently reduced in choline than in manganese deficiency. This was interpreted to be correlated with the slightly greater pathology observed in the choline deficiency. These investigators comment that the changes in manganese and choline deficiencies are so similar as to warrent postulation that the effects are mediated through some common biochemical system which presumably was enzymatic in nature. As compared to other pathological changes induced by vitamin or mineral deficiencies, Wolbach and Hegsted comment that the changes in the epiphyseal cartilage during perosis, because of the defective matrix formation, have a remote resemblance to the effect of vitamin C deficiency, although, in perosis, there was no evidence that the formation of intercellular substances had been affected, as occurs so dramatically in scurvy. In common with rickets, there was a failure of maturation of the epiphyseal cartilage cells in perosis, but all cartilage cell sequences were affected, and calcification of bone matrix was not suppressed.

The embryos hatched from eggs laid by manganese-deficient chicks were observed to have an incidence of chondrodystrophy.[128]

Copper Deficiency

Davis[129] describes the effect of copper deficiency in young cattle as being typical of rickets, with a beading of the ribs and an enlargement of the ends of the long bones. Eventually, the bones become very fragile and there is a high occurrence of broken bones, particularly of the ribs, legs, and thighs. Under severe conditions of copper deficiency among the adult cattle, as many as 10 per cent of all calves born are monstrosities, with enlarged heads, missing bones, and malformed bones. As copper deficiency becomes more severe, the females fail to come in heat and the males become completely sterile.

Teague and Carpenter[130] reported a sign of copper deficiency in pigs which outwardly resembled rickets. There was a lack of rigidity in the leg joints. The hocks became excessively flexed and forced the animals to assume a sitting position. The forelegs showed various degrees of crookedness. Neither chemical determinations of the bones nor histopathologic observations were reported.

Fluorine

No studies have been reported in which an acute fluorine deficiency was produced in experimental animals. The reason for this is the ubiquitous presence

of fluorides in foods and ration ingredients. Extensive surveys of the fluoride content of more than 130 foods are available. The majority of foods, such as vegetables, meats, cereals, and fruits contain 0.2 to 0.3 parts per million (ppm.) of fluorides. Outstanding exceptions to this lower range are the seafoods, the edible portions of which contain 5 to 15 ppm. of fluoride, and tea leaves, which contain 75 to 100 ppm. of fluoride. A cup of tea will supply approximately 0.12 mgm. of fluoride. Reliable analyses of the fluoride contribution by foods to the human diet from as distant areas as Toronto, Minneapolis, and Washington, D. C., indicate that the average diet supplies anywhere from 0.18 to 0.56 mgm. of fluoride daily without the use of unusual amounts of either seafoods or tea.[131-133]

We do not know whether there is any effect of insufficient dietary amounts of fluorides on the growth and development of teeth, but there is a large body of data pertaining to the development of, and later caries-resistance of the teeth.

As early as 1874, Erhardt[134] and, again, in 1892, Crichton-Browne[135] suggested that fluorides were important in the maintenance of teeth. The latter believed that his contemporaries consumed too little fluorides. He based this hypothesis on the increasing tendency to consume white bread and other highly refined foods which were lower in fluorides than whole grains. He strongly recommended reintroduction into the diet of appropriate amounts of fluorides. Little was done to examine the merit of these postulates. One of the first convincing evidences of such a relationship was provided by Bunting and his coworkers, in 1928, who reported the results of a survey in Minonk, Ill.[136] The amount of tooth decay in children born and reared in this community was much less than in children who moved to Minonk after tooth development was complete. At the time of the survey, the investigators recognized that this striking difference was related to the water supply, but the active agent was unknown. Later it was found that the drinking water contained 2.5 ppm. of fluorides.

In 1939, more exact information was given by Dean and his collaborators[137] as a result of a survey of 1581 children in four communities in the Illinois area, where the water contained varying amounts of fluorides. Later, a more comprehensive survey was described for 4,425 children from 13 cities in 4 states.[138] The data from the latter study are presented in TABLE 1, in terms of the number of decayed, missing, or filled permanent teeth, observed in the 12- to 14-year-old children. Where the water contained 1 ppm. of fluorides or more during tooth development, the children had a much lower incidence of tooth decay than children in nearby communities, where the water contained appreciably less than 1 ppm. of fluorides. These findings have been corroborated by investigators in other areas of the United States, as well as in Canada, England, South Africa, Ukraine, Italy, Greece, and Hungary. Deciduous teeth, likewise, have been shown to benefit when waters containing these amounts of fluorides were available during tooth development.[139] Adults in the United States, Argentina, England, and Hungary who had this exposure to naturally borne fluorides during tooth development likewise have a low incidence of tooth decay.[140-143] On the basis of these and many other studies, there can no longer be any doubt that the consumption of drinking water containing 1 or more ppm

TABLE 1

A COMPARISON OF THE FLUORIDE CONTENT OF THE DRINKING WATER AND THE AMOUNT OF TOOTH DECAY AMONG 4425 CHILDREN, 12 TO 14 YEARS OF AGE IN 13 CITIES FROM 4 STATES

(Based on Dean *et al.*[138])

	Fluoride content (ppm)	No. of children examined	Children with no tooth decay (per cent)	Average no. of diseased teeth per child
Colorado Springs, Colo.	2.6	404	28.5	2.5
Galesburg, Ill.	1.9	273	27.8	2.4
East Moline, Ill.	1.2	152	20.4	3.0
Kewanee, Ill.	0.9	123	17.9	3.4
Pueblo, Colo.	0.6	6.14	10.6	4.1
Marion, Ohio	0.4	263	5.7	5.6
Lima, Ohio	0.3	454	2.2	6.5
Middletown, Ohio	0.2	370	1.9	7.0
Zanesville, Ohio	0.2	459	2.6	7.3
Quincy, Ill.	0.1	330	2.4	7.1
Portsmouth, Ohio	0.1	469	1.3	7.7
Elkhart, Ind.	0.1	278	1.4	8.2
Michigan City, Ind.	0.1	236	0.0	10.4

of naturally borne fluorides, throughout the period of tooth development, confers a significant and prolonged caries-resistance.

The fluoride content of teeth developed in areas where different amounts of fluoride were present in the water supply closely parallels the amount of fluorides in the water.[144] Where the drinking water contained 0.0 to 0.3 ppm. of fluorides, as in Washington, D. C., the teeth of the native continuous residents had approximately 0.010 per cent of fluorides in the enamel and 0.024 per cent in the dentin. Where the water supply contained 1.0 to 1.2 ppm. of naturally occurring fluorides, as in Aurora, Ill., the teeth of comparable residents contained 0.014 per cent of fluorides in the enamel and 0.036 per cent in the dentin. Presumably the caries-resistance of the teeth is somehow related to their fluoride content.

Since the inorganic fluorides introduced into water supplies by nature had proven to be so effective, the next step was to determine whether the introduction of comparable inorganic fluorides into low fluoride waters would be of equal value. The first survey was begun at Grand Rapids, Mich., in January 1945, where the fluoride content of the water supply was increased to 1.2 ppm., under the joint sponsorship of the United States Public Health Service, the University of Michigan, and the Michigan State Department of Health. Muskegon, Mich., served as the control low-fluoride city. Soon after this, surveys were begun in several other cities. Some of the impressive data which are now available for the older surveys are presented in TABLE 2.[145] The over-all analysis of these data unquestionably indicates that the dental caries incidence in teeth formed during the survey period was on the average about 50 per cent lower than the caries incidence in otherwise comparable teeth formed prior to the increase in fluoride content of the water supply. As would be expected, the greatest benefits were in the youngest age groups. The similarity of the data

TABLE 2

REDUCTION IN TOOTH DECAY OBSERVED IN VARIOUS FLUORIDATION STUDY PROJECTS
(Based on Sognnaes *et al.*[145])

Community	Fluoridation		Age group	Reduction in decay*
	Date started	Report period		
		(*yr.*)	(*yr.*)	(*per cent*)
Grand Rapids, Mich.	Jan. 1945	8	6	70.8
			7	52.5
			8	49.2
			9	48.1
			13	39.7
Brantford, Ont.	June 1945	7	6	59.4
			7	69.5
			8	51.5
			9	46.2
			13	32.9
Newburgh, N. Y.	May 1945	7	6	69.4
			7	67.8
			8	40.4
			9	51.4
Evanston, Ill.	Feb. 1947	4	6	73.6
			7	56.4
			8	35.4
Sheboygan, Wis.	Feb. 1946	6	9–10 (4th grade)	35.3
			12–14 (8th grade)	29.7

* Decayed, missing, and filled teeth.

from the several survey communities is amazing. No comparable decreases in dental caries incidence were noted in the children of the nearby cities where the fluoride content of the communal water supplies was not increased.

The ingestion of excessive amounts of fluorides during tooth development results in mottled enamel. As the fluoride content of the drinking water increases appreciably beyond 1 ppm., the percentage of individuals affected and the severity of the mottling increases, until, at levels of 8 to 10 ppm., a high percentage of the individuals who grow up in the area have mottled enamel of such severity that it is esthetically disfiguring. At fluoride levels from 2.5 ppm. on up, the water supply is in need of attention, either by the development of a new source, by adequate dilution with low fluoride waters, or by the removal of the fluorides. However, on the basis of these epidemiological surveys, fluorides contributing around 1 ppm. of fluoride to a water supply are considered to be of no detrimental public health significance with respect to the causation of mottled enamel.

Other abnormalities than those caused in the developing teeth by excess fluoride ingestion have been sought in various investigations in the United States.

Probably the most important and extensive surveys about general systemic influences of fluoride ingestion were those made in 1943 and 1953 in Bartlett and Cameron, Texas.[146] The former community had a water supply which contained approximately 8 ppm. of fluoride, whereas the latter community, sit-

uated some 30 miles distant, had a water supply with essentially no fluorides. In 1943, a series of inhabitants who had resided at least 15 years in each community was selected at random and carefully examined by skilled physicians. A total of 116 were examined in Bartlett, and 121 in Cameron. These individuals ranged from 15 to 68 years of age in 1943; 57.8 per cent of the Bartlett participants and 47.2 per cent of those from Cameron were over 55 years of age. X rays were made of their skeletal system and full case histories taken. Except for the individuals who died in the course of the 10 years between the examinations, the same individuals were examined or contacted again in 1953.

The data obtained in these surveys indicated that there was no significant difference in any phase of health between individuals in the one community and the other, with two exceptions. Many of the individuals who had resided in Bartlett during childhood had severely mottled teeth. In addition, a slightly higher incidence of cardiovascular disease was observed in Cameron. In all other regards, there were no detectable abnormalities which could be attributed to the different fluoride content of these two water supplies.

Over 3,000,000 individuals in the United States have consumed natural fluoride-bearing waters in excess of 1 ppm. for decades, and an additional 5,000,000 individuals have consumed amounts between 0.5 and 1.0 ppm.[147] Already, the fluoridation of community water supplies has been widely instituted throughout the United States and elsewhere in the world. As of May 1, 1954, fluorides were being added to the water supplies in 944 cities and towns in the United States with a total population of nearly 17,000,000.[148] Many other communities are in some phase of equipment purchase and installation. Since then, the Chicago City Council has approved fluoridation and has ordered its Department of Water and Sewers to have the program in operation by Jan. 1, 1955.[149] This water system serves about 3,600,000 residents of the city and and an additional 500,000 in adjacent suburbs.

There is an increasing amount of evidence that other minerals and certain ratios of minerals are responsible for changes in both the caries-resistance and chemical composition of teeth.[150-152] A great deal of investigation is necessary to elaborate fully upon these influences.

Conclusions

The area of nutrition concerning the development and calcification of the bones and teeth has been relatively little explored in comparison with the wealth of material there. Though there are classical descriptions of the major pathologic states in the bones and teeth which result from nutritional deficiencies, none of the relatively new scientific disciplines has been applied to any appreciable extent for the elaboration in more specific terms of what these classical pictures represent or the intimate details of why they have occurred. Likewise, there has been little attempt to evaluate with precise analytical procedures the effect that these deficiencies have upon the composition of the matrix or the bone salts formed in the chronic or prolonged deficiency or in the recovery state. Sobel's outstanding studies on the relation of altered serum concentration of carbonate, phosphate, and calcium to the composition of the "bone salt" point

with clarity to the potentialities for research in this area. His observation that there was a characteristic species difference in normal phosphate-carbonate ratios in the "bone salt" of the bones and teeth of white rats and cotton rats is of particular interest. Is it too farfetched to suggest that, within a species of rodent there may be differences in the composition of the "bone salt" deposited in different strains, even though they are maintained for generations on the same diet? Does this suggestion offer a possible answer to the wide differences in caries-susceptibility between the various strains of the common laboratory rat?

There is one conclusion which seems to be particularly clearly indicated: where cells of comparable origin exist in bones and teeth and other areas of the body, or where comparable compounds are deposited by these cells even though in different forms of orientation, the reactions in the structures to a given nutritional deficiency during development are closely analogous despite the different functional differentiations of the structures. In view of the striking similarities between the calcification in bones and the components of the teeth in either normal or nutritional deficiency states, there are a number of strong reasons which suggest the increased use of the continuously growing rodent incisor as a site for a variety of studies on metabolic influences upon calcification. Probably the most obvious reason for the use of this organ in these studies is its ready accessibility for sampling without appreciable interference with the well-being of the animal. What may well prove to be the greatest reason for the use of the incisor of the rat in these studies is the fact that the overlapping effects of bone formation and remodelling sequences which lead to many difficulties of sampling and interpretation in bone physiology do not exist in teeth.

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HORMONAL INFLUENCES ON SKELETAL GROWTH

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The principal purpose of reviewing and discussing some of the clinical aspects of disturbed skeletal growth is to draw attention to various gaps in the knowledge of osseous development which require further investigation.

Developmental Changes in the Skeleton

In examining a patient with stunted growth, it is important to determine whether there is generalized impairment of bone growth in all directions, so that infantile proportions persist, or whether there are still the differences in growth potentials which are responsible for the changing of proportions that normally occurs with advancing age. This question is most easily determined by measuring the ratio of the upper and lower skeletal segments and observing the development of the naso-orbital configuration. Patients with hypothyroidism retain infantile proportions, whereas those with hypopituitarism or with primordial dwarfism, even when they are equally stunted in height, usually show more mature skeletal proportions approximately commensurate with their age.

The time of ossification of the carpal and tarsal cartilages and the epiphyseal centers of the long bones is another index of skeletal maturation. The epiphyseal development is not related to the changes in skeletal proportions just mentioned. It is markedly retarded in both hypothyroidism and hypopituitarism, and may be retarded to some extent in many other conditions. The more the epiphyseal development is retarded, the greater is the opportunity for further growth.

The height which an individual attains depends upon both the rate of growth and the duration of growth before epiphyseal fusions occur. The exact mechanism of fusion is not understood and it should be studied further. One might expect that the union is due to cessation of growth of the epiphyseal cartilage, caused possibly by diminution of the secretion of pituitary growth hormone after the age of puberty. In hypopituitary dwarfs, however, in whom growth comes almost to a standstill, even after the epiphyseal centers have fully ossified, these centers fail to fuse with the ends of the bones. It has been stated that androgen or estrogen may be responsible for epiphyseal fusions. How these fusions occur is not known. When testosterone is given to a hypopituitary sexually infantile male with marked delay in the epiphyseal ossification, there is rapid growth of the long bones and of the epiphyses themselves, and eventually the fully ossified epiphysis fuses with the shaft. The pattern is the same as that which occurs in normal adolescence. The effects of estrogen on epiphyseal fusions are even less well understood.

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TABLE 1
CAUSES OF STUNTED GROWTH
(Wilkins, 1953)

I. *Bone diseases*
  (A) Chondrodystrophy—various types
  (B) Rickets, all types
  (C) Osteogenesis imperfecta
  (D) Disease of the spine, such as tbc. caries
II. *Nutritional or metabolic disorders*
  (A) Celiac disease and cystic fibrosis of pancreas
  (B) Chronic renal disease
  (C) Hepatic insufficiency
  (D) Hurler's syndrome of gargoylism (dysostosis multiplex)
  (E) Nutritional defects and chronic infections
III. *Circulatory disorders with anoxemia*
  (A) Congenital malformations of the heart
  (B) Extensive chronic pulmonary disease
IV. *Endocrine disturbances*
  (A) Hypothyroidism
  (B) Sexual precocity with early epiphysial fusion
  (C) Hypopituitarism
  (D) Mixed types
V. *Delayed adolescence with retarded growth spurt*
VI. *Primordial or genetic dwarfism (constitutional)*
  (A) Familial
  (B) Sporadic
  (C) Syndrome of ovarian agenesis and dwarfism
VII. *Progeric types of dwarfism*
  (A) Hutchinson-Gilford type
  (B) Cockayne-Neill type

Causes of Stunted Growth

TABLE 1 gives a classification of the causes of stunted growth. In some of these conditions, such as the chondrodystrophies, there is probably some inborn defect in the enzyme systems or chemical composition of the cartilage and bone which is responsible for the failure of the normal orderly growth of cartilage and its conversion into bone. The nature of this defect is not known. In osteogenesis imperfecta, in which deformities and stunted growth may result from multiple fractures, Follis has shown that there is an abnormality of collagen fibers, not only in bone, but in the skin, in the sclerae, and throughout the body. In dysostosis multiplex (Hurler's syndrome of gargoylism), in addition to skeletal changes resembling chondrodystrophy, there is apparently an inborn abnormality of metabolism causing the cells of the liver, spleen, cornea, and many other tissues to be infiltrated with some abnormal material thought to be a mucoprotein. Probably most of the intestinal, hepatic, nutritional, or cardiorespiratory disorders listed impair skeletal growth through their effects on the absorption or transportation of substances required for the normal organic metabolism of bone. Renal diseases that cause impaired phosphate excretion with elevated serum P and low serum Ca, lead to secondary hyperparathyroidism with characteristic bone changes. The renal tubular disorders that lead to diminished reabsorption of P and low serum P may cause rickets or osteomalacia. On the other hand, renal impairment sometimes causes marked dwarfing without apparent abnormalities in bone structure.

In addition to the various diseases mentioned, one must also exclude primordial or genetic types of dwarfism and constitutional delay in growth and adolescence, before attributing stunted growth to a specific endocrine defect.

Hormonal Influences on Skeletal Growth

The hormones which exert the most apparent effects on skeletal and other somatic growth are (1) the *pituitary growth hormone;* (2) *thyroid hormone;* and (3) *androgen of testicular or adrenal origin.* All three of these hormones have been shown, under proper conditions, to increase both protein anabolism and skeletal growth. Comparatively little is known concerning their exact mode of action on bone, and it is probable that each of these hormones operates in a different manner. Their relative importance may differ in the various stages of growth during childhood and adolescence. In contrast to these hormones which stimulate growth, excessive secretion of *adrenal corticoids* exerts an inhibitory effect which is observed in Cushing's syndrome. This effect must be taken into consideration in the use of cortisone for the treatment of children during the growth period.

Some of the differences in the action of thyroid, growth hormone, androgen, estrogen, and corticoids are illustrated by clinical observations to be discussed. Time will not permit a consideration of the effects of *estrogen* and *parathyroid hormone* on the structure of bone.

Hypothyroidism. When hypothyroidism occurs during childhood, it always causes retardation of growth and all the processes of development. Not only is there marked decrease in the rate of skeletal growth, but also the changes in proportions which are associated with skeletal maturation do not occur. Accordingly, the hypothyroid dwarf retains infantilistic skeletal proportions and naso-orbital configuration, in marked contrast to the hypopituitary dwarf who may have proportions corresponding to his chronologic age. The clinical picture found in a patient with hypothyroidism varies with (1) the age at which thyroid deficiency began, (2) its duration before treatment, and (3) the degree of deficiency. Accordingly, the untreated congenital cretin shows marked infantilistic characteristics that are not shown by a patient who becomes hypothyroid later in childhood after a more advanced level of development has been attained.

In addition to stunted growth, there is always marked delay in the time of ossification of the epiphyseal centers. This delay is not unique in hypothyroidism or diagnostic of it. Equally marked retardation of the "bone age" is seen in some hypopituitary dwarfs. In fact, delayed ossification of cartilage is found in many conditions which cause severe malnutrition or circulatory impairment. We have seen it occur unilaterally in hemiplegics who have poor circulation on the affected side. Some children without any endocrine disorder are "constitutionally" slow in their growth and epiphyseal development and in the onset of puberty. When retardation of growth and epiphyseal ossification is due to hypothyroidism, the administration of thyroid hormone always causes a rapid and spectacular acceleration. In hypopituitarism and other conditions, thyroid therapy has no appreciable effect. It is of interest that, although the hypo-

pituitary dwarf shows no response to thyroid, testosterone will cause a spurt of growth and epiphyseal development.

The most characteristic and specific skeletal abnormality in hypothyroidism is that known as "*epiphyseal dysgenesis.*" Normally, ossification of the carpal and tarsal bones and of the epiphyseal cartilages first appears radiologically as a single small center which grows larger in a regular manner. In hypothyroidism, not only is the time of ossification delayed, but, when eventually it occurs, it begins in multiple small centers scattered over a large area of the cartilage. These centers grow large and coalesce, causing a stippled, porous, fluffy, or fragmented appearance. In patients with congenital hypothyroidism who have remained untreated for a long time, practically all the cartilaginous centers may be involved, although the abnormality is most readily seen in the larger centers, such as the head of the femur. At the time that treatment is begun, many of the centers may be entirely uncalcified and not visualized in X rays, while some of the early centers show typical dysgenesis. Under treatment, ossification is greatly accelerated and all of the centers which were delayed during the hypothyroid state show the abnormality when they first appear (FIGURES 1 and 2). The centers which normally were not due to ossify during the period before treatment was begun do not show abnormal structure.

The findings described suggest that, during the hypothyroid state, the cartilage may undergo an alteration preparatory to ossification over a wide area, but that the further steps of calcification and bone formation are impeded. When thyroid medication is given, this large zone of altered cartilage promptly undergoes calcification and osteoid invasion in an irregular fashion. This hypothesis

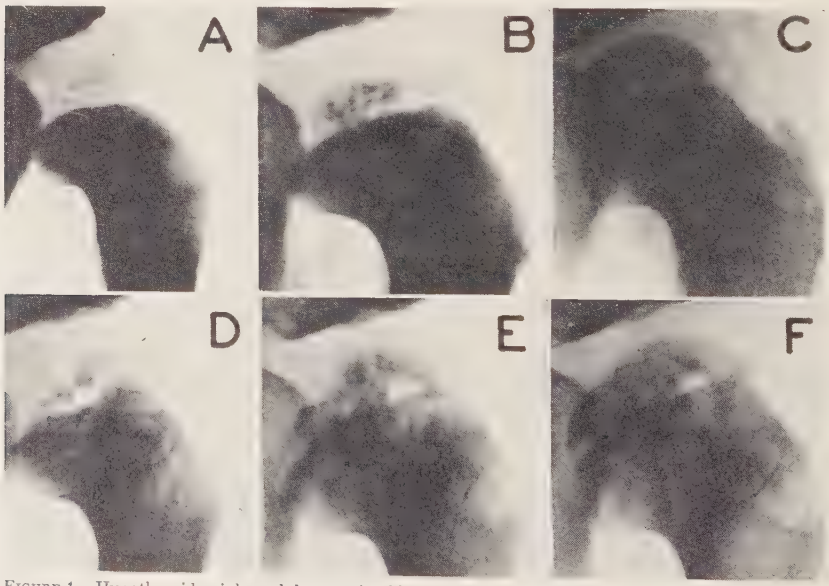

FIGURE 1. Hypothyroid epiphyseal dysgenesis of hip.
(A, B, C) Hip of a 6-year-old cretin, 2, 5, and 22 months after beginning thyroid treatment.
(D, E, F) Hip of a 9-year-old cretin before treatment, and 7 and 12 months after treatment (from Wilkins, 1950).

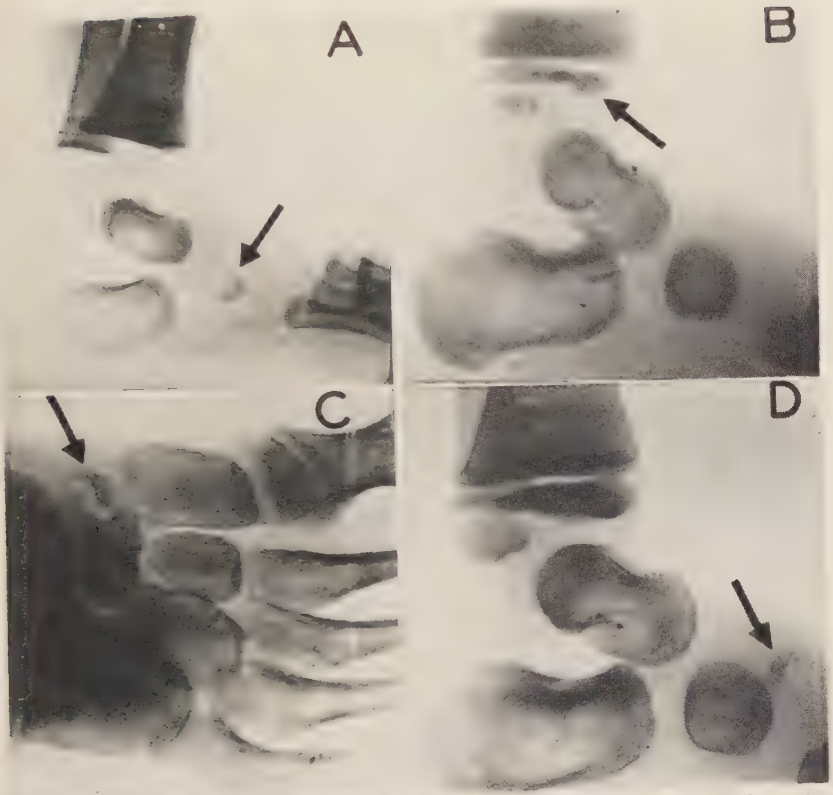

FIGURE 2. Hypothyroid epiphyseal dysgenesis of various bones. These centers appeared under treatment. Note the line of arrested growth and periosteal zone of new bone in the calcaneous and talus (from Wilkins, 1950)

seemed to be borne out to some extent by observations we made many years ago on kittens thyroidectomized on the seventh day of life (FIGURE 3). In the control litter-mates, the ossification in the center of the cartilage was preceded by a narrow zone in which the cartilage cells were large and swollen, and the matrix deeply basophilic. In the thyroidectomized animals, the zone of swollen cartilage cells and basophilic matrix became very large before there was any formation of osseous tissue, and its invasion by capillaries and osteoid tissue seemed to occur irregularly. More studies are desirable on the effects of thyroid on the chemistry and enzyme systems of the cartilage and bone and on the growth of the capillaries and their invasion into the cartilage.

Hypopituitary Dwarfism versus "Primordial Dwarfism" versus Delayed Adolescence

The diagnosis of hypopituitary dwarfism is usually very difficult before the adult age. When a dwarf remains sexually infantile and has a low excretion of urinary gonadotropins, one may conclude that there is a pituitary defect involving the secretion of both growth hormone and gonadotropins. On the

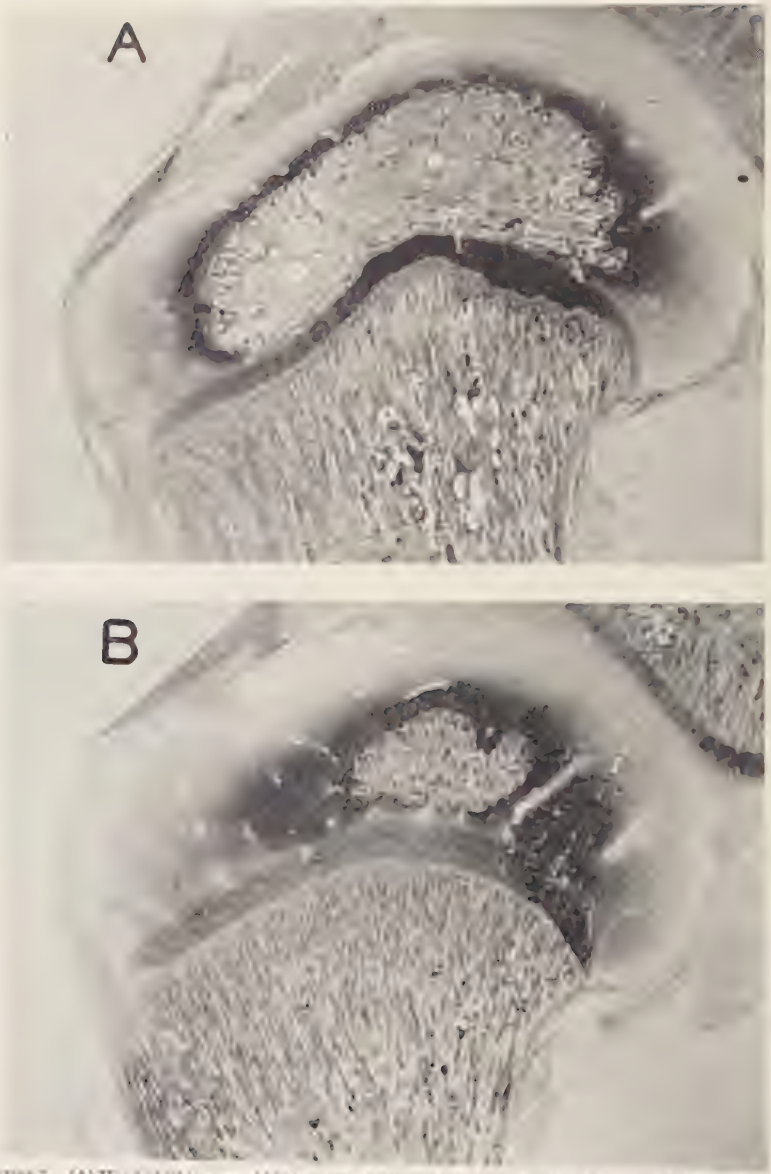

FIGURE 3. (A) Head of tibia normal kitten, age 21 days. Area of ossification is surrounded by only a narrow zone in which cartilage has undergone preparatory change.
(B) Head of tibia in kitten thyroidectomized on 7th day and killed on 21st day. Small irregular area of ossification surrounded by large dark area in which cartilage cells are swollen and matrix is deeply basophilic (from Wilkins, 1950).

other hand, when a dwarfed individual shows entirely normal sexual maturation and presents no evidence of any other endocrine defect we prefer to apply the term "primordial dwarfism," admitting that the growth impairment may be due to a generalized constitutional trait, possibly of genetic origin, rather

than to a specific deficiency of growth hormone without other pituitary involvement. In addition, there are individuals who are slow in all their growth and development throughout childhood and have a late onset of puberty, but eventually mature into entirely normal but sometimes short adults. These are considered instances of "constitutional" delay in growth and adolescence.

In childhood, it is difficult to distinguish between hypopituitary dwarfism, primordial dwarfism, and constitutionally delayed growth and development. These three types of individuals present no characteristic differences in their habitus. Some hypopituitary dwarfs are slender and have small bones and thin, fine features. Others are stocky and have round, chubby faces. Unlike children afflicted with hypothyroidism, both hypopituitary and primordial dwarfs may have ratios of upper and lower body segments that are relatively normal for their ages; and they do not show the other markedly infantile characteristics seen in hypothyroidism. Hypopituitary dwarfs generally have marked retardation in the time of ossification of their epiphyses, which may be as great as in hypothyroidism. In contrast, the primordial dwarf usually has an essentially normal bone age. One might ask whether the osseous retardation might be due to some degree of secondary hypothyroidism. This condition seems unlikely in view of the fact that the administration of thyroid does not cause an appreciable acceleration of epiphyseal development or of growth (FIGURE 4). Furthermore, epiphyseal dysgenesis does not occur in hypopituitary dwarfs. Epiphyseal fusions do not occur in hypopituitary dwarfs or appear very late in life, but normal fusion takes place in primordial dwarfs. The bones remain small and delicate in pituitary dwarfs, probably due to relatively poor muscular development, but there is no true osteoporosis. The facies remain juvenile in the sexually infantile patients, but normal adult development of the features takes place when a primordial dwarf matures sexually. Some of the differences between various types of dwarfs are recorded in TABLE 2.

The "syndrome of ovarian agenesis and stunted growth" is of considerable interest. In females with this condition, the ovaries do not develop beyond the stage of the primitive genital streak and contain no ova or germinal epithelium. Estrogen is completely lacking, but moderate amounts of adrenal androgen are secreted. Accordingly, the breasts, labia minora, and female genital tract remain infantile, but sexual hair appears. Pituitary gonadotropins are greatly increased, whereas they are low or absent in hypopituitary dwarfs. Patients with the syndrome are small from birth or early childhood. They have no adolescent spurt of growth, and eventually they attain a height of only 52 to 58 inches. The dwarfism resembles the primordial rather than the hypopituitary type. Epiphyseal ossification and fusion occur normally. The facies develop into the adult type. The dwarfing cannot be explained by the deficiency of estrogen or any other hormone, since girls whose ovaries are removed before puberty usually grow very tall and have eunuchoid proportions. The short stature is probably a "genetic" or congenital anomaly, as is the agenesis of the ovary. In addition to the dwarfing, there is often fairly marked osteoporosis of the metaphyses of the long bones and of the carpal and tarsal bones. Many other congenital anomalies, including minor abnormalities of the skeleton, occur in this

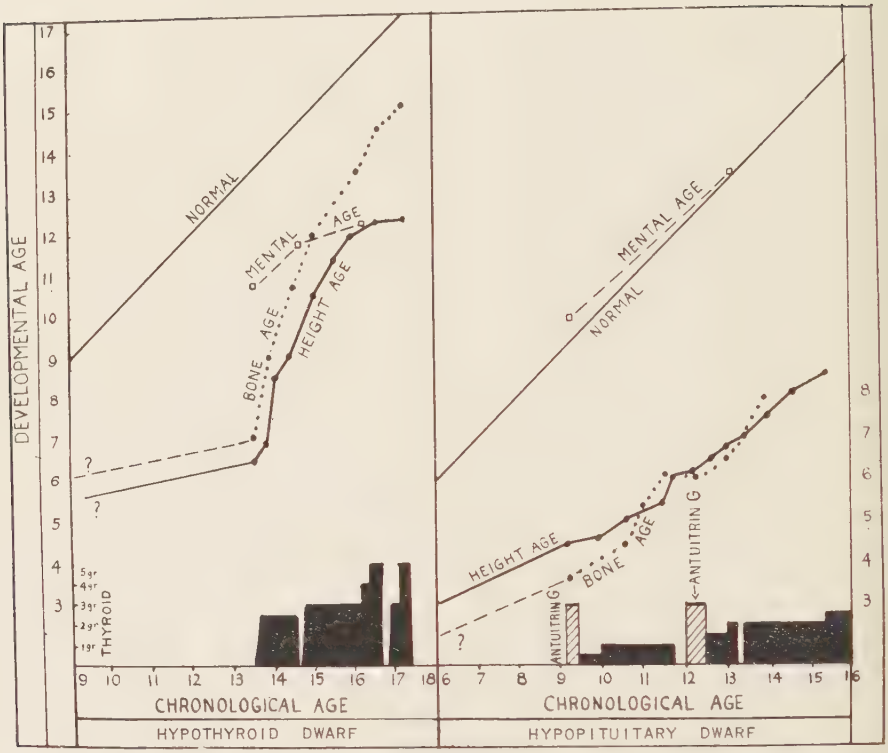

FIGURE 4. Retardation of epiphyseal ossification occurs not only in hypothyroidism but also in hypopituitarism and other conditions. Thyroid therapy causes rapid acceleration of growth and development in cases of hypothyroidism but not in hypopituitarism and other conditions (from Wilkins, 1950).

syndrome. The most striking of these are webbed neck and coarctation of the aorta.

Treatment of dwarfism. Pituitary growth hormone, although effective in rats, has not succeeded in stimulating growth in human beings. In fact, hormone prepared by the Russell and Wilhelmi technique and an electrophoretically pure preparation made by Li and Evans have been shown by a number of workers to cause nitrogen loss and to have other toxic effects. Whether this phenomenon was due to contamination with small amounts of thyrotropic hormone, to denaturization by lyophilizing, or to a species difference with antibody formation is not known. Vitamin B_{12} has no effect on skeletal growth.

Thyroid medication causes spectacular acceleration of growth and epiphyseal ossification in hypothyroid dwarfs, but not in other types. In the hypothyroid patient, it increases protein anabolism with retention of N, K, P, and Ca.

In prepuberal boys, the administration of chorionic gonadotropin causes an adolescent growth spurt by stimulating testicular development and androgen secretion. We believe that such treatment should be given only when there is a very long delay in the onset of puberty causing severe psychologic problems.

Except for the effects of thyroid in cases of hypothyroidism, testosterone is

TABLE 2
SOME DIFFERENCES IN TYPES OF DWARFISM
(Wilkins, 1953)

	Degree of dwarfing	Epiphysial ossification and fusion	Skeletal proportions	Features	Sexual development	Special characteristics
Hypothyroidism	+++	Marked retardation	Infantile	Infantile "cretinoid"	Late with incomplete maturation	Epiphysial dysgenesis (stippling), high cholesterol, other signs
Delayed adolescence	+	Moderate retardation	Normal	Immature	Retarded but eventually normal with late growth spurt	None
Pituitary dwarfism	+ to +++	Retardation	Normal	Immature	Remains infantile	F.S.H. low, 17-K.S. low. Prolonged hypoglycemia with insulin
Primordial dwarfism	+ to +++	Normal	Normal	Mature	Normal maturation	Otherwise normal
Ovarian agenesis	+	Nearly normal	Normal boyish	Mature	Female organs infantile. Sexual hair present	F.S.H. high, 17-K.S. slightly low. Osteoporosis, other genetic defects
Sexual precocity	+	Premature	Short lower segment	Precocious mature	Precocious	F.S.H. normal, 17-K.S. high or normal. Rapid growth with premature ep. fusion
Progeria	+++	Normal	Mature	Old wizened	?	Slender emaciated bird-like features, arteriosclerosis, scleroderma (?)

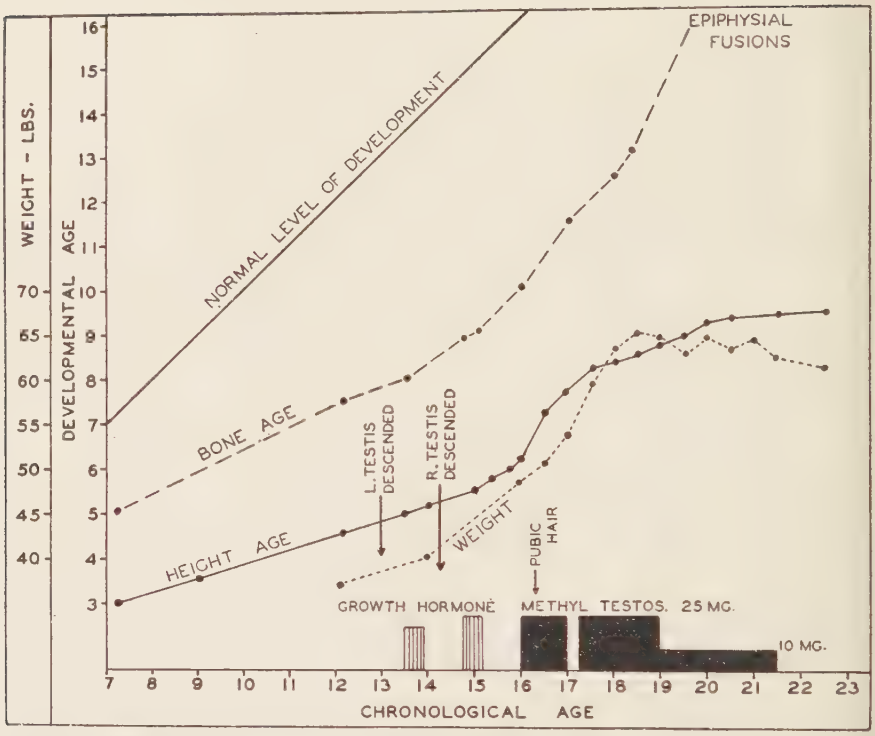

FIGURE 5. Effects of methyltestosterone on growth and development of hypopituitary dwarf (from Wilkins 1950).

the only hormone causing a marked acceleration of skeletal growth. This acceleration occurs both in normal prepuberal children and in hypopituitary dwarfs. The pattern of growth is similar to the spurt of growth which occurs normally in adolescence. Simultaneously with growth in the length of the long bones, there is development of the epiphyses. Premature fusion of the epiphyses does not occur, but eventually they fuse, and growth ceases (see FIGURE 5). In male hypopituitary dwarfs with marked retardation of bone age, testosterone may cause a growth of as much as five inches in the first year with slower growth during the next year or two, so that a total increment of seven to nine inches may be attained. The effect of testosterone on growth is probably due to its action in increasing the protein anabolism of all body cells and is not mediated through the pituitary. Attempts have been made to find a steroid having a similar protein anabolic effect without virilizing action. Although this claim has been made for methylandrostenediol, it now seems that this compound is a weak androgen and that, if given in large amounts to cause growth, it also causes virilization.

Estrogens do not have the growth-stimulating effects of androgens. When given to female hypopituitary dwarfs, no nitrogen retention or skeletal growth occurs. When testosterone is given, in addition to estrogen, the same effects

on growth are noted as in the male. In patients with ovarian agenesis, estrogen alone may cause a slight spurt of growth. This response is probably due to its effect in increasing secretion of adrenal androgen, as evidenced by increased urinary 17-ketosteroids and sexual hair.

Effects of androgen on growth in cases of sexual precocity. Both adrenal and testicular androgens seem to have the same effect on growth and protein anabolism. Accelerated growth and osseous development are seen in males with the constitutional type of sexual precocity, males with congenital adrenal hyper-

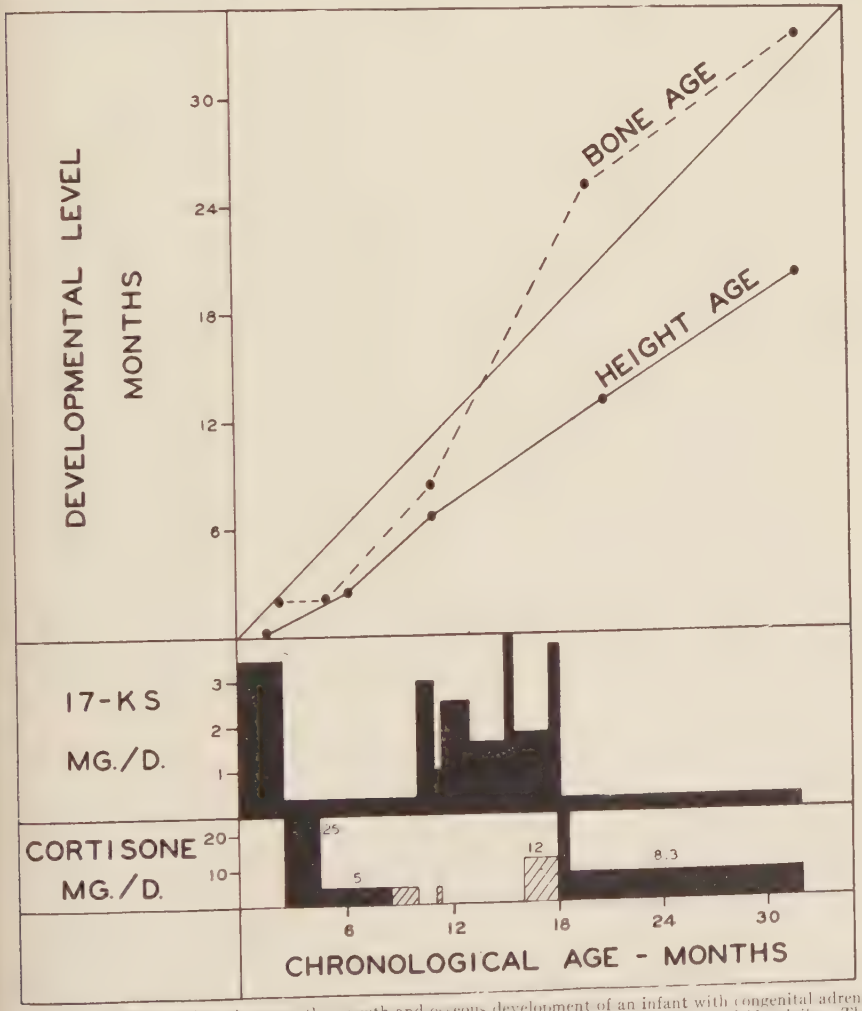

FIGURE 6. Effects of cortisone on the growth and osseous development of an infant with congenital adrenal hyperplasia. During the first period of treatment the patient received 25 mgm. of cortisone (Merck) daily. This treatment suppressed the 17-ketosteroid excretion, but was excessive and caused the appearance of Cushinglike symptoms and inhibited osseous development. On reduction of the dose of cortisone to 5 mgm. daily, the 17-ketosteroids remained suppressed and normal growth and osseous development took place. When cortisone was omitted, the 17-ketosteroid output rose and osseous development was accelerated due to excessive androgen. When cortisone therapy was resumed at the proper level (8.3 mgm. daily), growth and osseous development proceeded at approximately normal rate.

plasia or tumor, and female pseudohermaphrodites with congenital adrenal hyperplasia. All of these patients grow with great rapidity during early childhood. Their epiphyseal development becomes even more advanced than the height. By the age of four years, the height age may be seven years and the bone age 12 years. By the age of six or eight years, all the epiphyses may have fused, and growth ceases before full adult height is attained. The growth of the extremities is less rapid than the trunk so that these patients have proportions suggestive of chondrodystrophy.

Effects of adrenal corticoids. In Cushing's syndrome, the predominant metabolic picture is opposite to that of the adrenogenital syndrome. Instead of excessive protein anabolism and accelerated growth due to androgen, there is excessive gluconeogenesis due to the secretion of corticoids and inhibition of protein anabolism. Children with Cushing's syndrome are somewhat retarded in growth and the epiphyseal development is not accelerated.

In congenital adrenal hyperplasia, we are now able to suppress the androgenic hyperactivity of the adrenal by the administration of cortisone in small amounts which are equivalent to the physiologic requirements. This therapy puts at rest the patients' adrenals, which secrete an abnormal pattern of androgenic steroids. If treatment is started before epiphyseal fusions have occurred, the excessive growth and osseous development are prevented. In treating young children with adrenal hyperplasia, it is most important to adjust the maintenance dose of cortisone to levels which suppress the excessive androgenic activity of the adrenal but do not exceed the physiologic requirements for gluconeogenetic hormone. If these requirements are exceeded, protein anabolism and growth are inhibited. Accordingly, in adjusting the maintenance doses of cortisone, it is important to follow not only the excretion of urinary 17-ketosteroids to determine whether the hyperactivity of the adrenals is adequately suppressed, but also the rates of growth and osseous development to be certain that the amount of cortisone is not excessive (see FIGURE 6). At present, we seem to be able to regulate the dosage so as to attain reasonably normal growth and development. The longest period of treatment is about three and one-half years, and it will be of great interest to follow the growth and development of these patients during the next ten years.

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STUDIES ON THE ROLE OF THE PARATHYROIDS IN CALCIUM AND PHOSPHORUS METABOLISM*

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The parathyroids constitute the major factor responsible for the constancy of the blood calcium concentration necessary for normal development and maintenance of the skeletal tissues. In recent years, this important hormonal activity has not been accorded the intensive and widespread research endeavor which it deserves, and it is encouraging to note that there are now indications of a new burst of investigational effort in this area.[1, 2] Here is an endocrine system in which the hormonal activity is still known only in the crude state; its chemical nature is still largely a matter for conjecture; the scope and mechanism of its actions are still incompletely defined; and its potential therapeutic applications await necessary prior advances in basic knowledge. It is an attractive field for further scientific exploration.

At the risk of slighting an important opportunity for a definitive review of the present status of knowledge about the parathyroids, we have chosen to present for your critical appraisal a summary of the largely unpublished work carried out in our laboratory during the past three years. In the interest of relative brevity, only scant mention will be made of pertinent observations made by other investigators.

The over-all direction of our investigation is toward the elucidation of the nature and mechanism of action of the hormonal activity of the parathyroids. Our major instrument in these studies has been the parathyroidectomized rat. Surgical excision of the parathyroids from the strain of albino rats we use (obtained commercially from the Holtzman Rat Company) is not difficult, and can be carried out speedily without obvious damage to the thyroid. There is, however, a certain amount of hemorrhage associated with the operation and, in a considerable proportion of cases, this bleeding may be extensive enough to result in death from loss of blood. In all cases, it was feared that the amount of blood lost might affect the concentrations of calcium and inorganic phosphate ions significantly. Accordingly, Doctor Greep, my collaborator in most of these studies, revived the procedure, originally introduced by Erdheim, of destroying the parathyroid tissue with an electrocautery. This procedure is a convenient one. It is performed under ether anesthesia with the aid of a dissecting microscope and requires only about two minutes time per rat. There is no hemorrhage and no mortality other than that directly attributable to the loss of the parathyroids. In our experiments, we have not been troubled by interference from functional accessory parathyroid tissue missed by the surgeon.

In a series of early experiments,[3] in collaboration with Oscar Iseri and Doctor Greep, male albino rats, 40 to 50 days old, were placed on a purified diet (modified from Shaw [4]) which is essentially free of calcium but otherwise nutritionally

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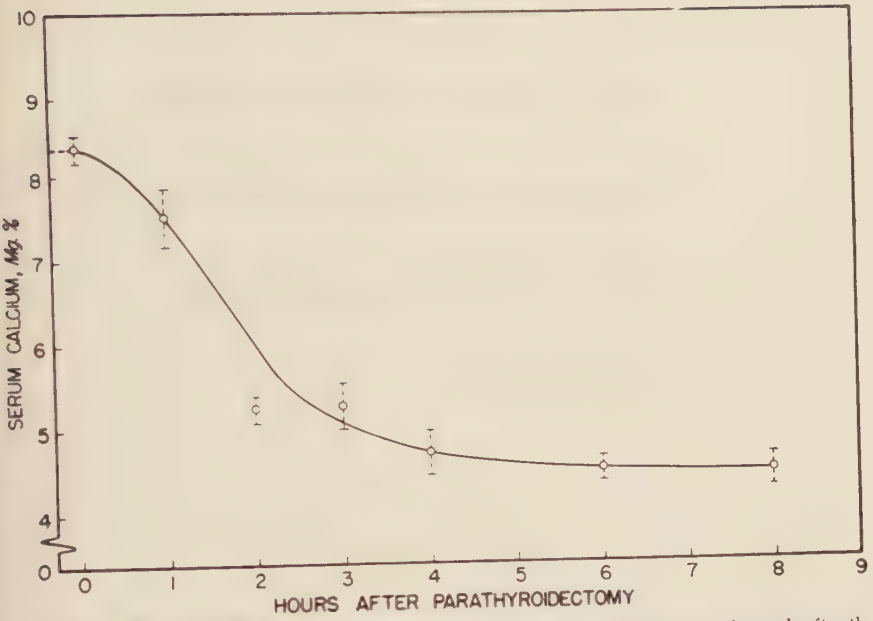

FIGURE 1. Effect of parathyroidectomy on serum calcium of male rats at varying time intervals after the operation. The rats had been maintained on a diet low in calcium for 10 to 20 days prior to parathyroidectomy. The vertical bars represent the standard errors. Number of rats: 6 to 38 for each point.

adequate, for 10 to 20 days, then parathyroidectomized at an age of 50 to 60 days. Groups of rats were bled by heart puncture at varying time intervals after parathyroidectomy, and the serum was analyzed for calcium by the method of Fiske and Logan.[5] The results are shown in FIGURE 1. The rapidity and extent of the fall in serum calcium were noteworthy. A significant fall had occurred within one hour after parathyroidectomy and, in two hours, the serum calcium had fallen almost to the minimum finally attained. Rats allowed to survive went into severe tetany and died between 6 and 10 hours after the operation. About 130 rats were used in these experiments, from 6 to 38 for each point. At the time of the operation, as a consequence of the dietary calcium restriction, the serum calcium was somewhat low, 8.3 mg. per cent on the average instead of the normal 10 mg. per cent. From the experiments of others[6] and from our own observations, we have inferred that the parathyroids were hyperactive and that the skeleton was hypocalcified at zero time. The status of these rats, then, at the time of parathyroidectomy, might be expected to influence the effects of parathyroidectomy, and this possibility will receive further consideration.

The fall in serum calcium observed in these experiments demonstrated that, prior to the removal of the parathyroids, these glands had furnished a hormone or hormones which were necessary to maintain the serum calcium at approximately 8.5 mg. per cent. The rapidity of the fall also indicated that the parathyroid hormone in the circulation responsible for maintenance of blood calcium had been rapidly excreted, destroyed, or otherwise inactivated. Even before

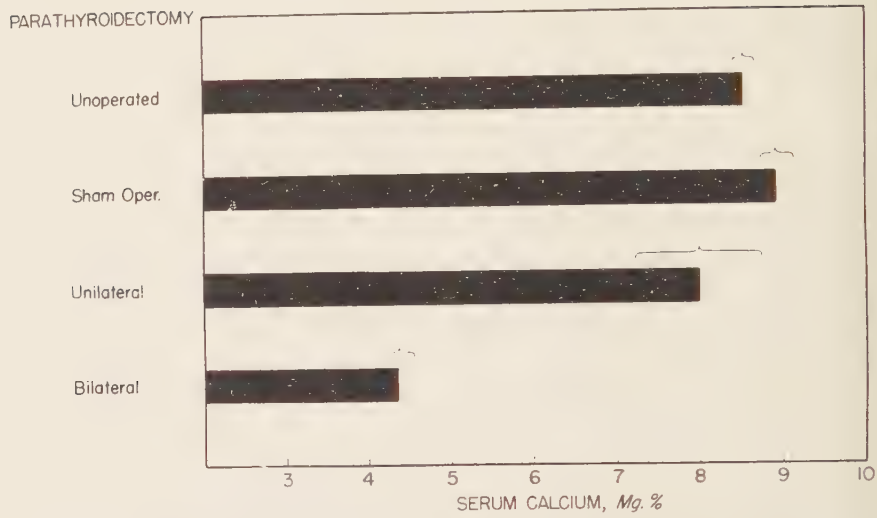

FIGURE 2. Serum calcium of rats unilaterally parathyroidectomized compared with serum calcium of rats unoperated, sham operated, and bilaterally parathyroidectomized. Blood was collected six hours after operation. Brackets represent standard errors.

parathyroidectomy, there was not enough hormone secreted to maintain the serum calcium at 10 mg. per cent under the adverse conditions imposed by the experiment. However, other experiments (FIGURE 2) have indicated that a marked reduction in the rate of hormonal secretion, such as the 50 per cent reduction which presumably followed unilateral parathyroidectomy, was insufficient to result in an important fall in the level of serum calcium. In this experiment, calcium-depleted rats were either sham-operated or unilaterally parathyroidectomized, and the serum calcium, six hours after the operation, was then compared with that of intact rats and rats bilaterally parathyroidectomized. Although the greater variability of the unilaterally operated group suggested a trend toward a depressed serum calcium, there was actually no significant difference from the intact and sham-operated groups.

In the first experiment (FIGURE 1), the serum calcium levels were already at tetanic levels two hours after parathyroidectomy, but actual tetany was not usually manifest much before 5 hours. From 6 hours on, tetany was obvious and the rats died between 6 and 10 hours after the operation. The values indicated at 8 hours on the graph comprise blood samples collected during severe tetany between 6 and 10 hours postoperatively and shortly before death would otherwise have supervened. The explanation for the delayed onset of tetany is an intriguing question, and it is suggested that studies of the mechanisms involved in hypocalcemic tetany might profitably be conducted with parathyroidectomized rats during the period which intervenes between the attainment of maximal hypocalcemia and the appearance of severe tetany.

The attractive hypothesis of Fuller Albright and Ellsworth[7] that the parathyroids regulate the blood calcium indirectly, by their effect on phosphate reabsorption by the kidney, is well known. Forty-three years ago, Greenwald[8]

discovered that parathyroidectomy in dogs was followed by a marked decrease in the urinary elimination of phosphate, and he and Gross later demonstrated[9] that the contrary effect, that is, increased excretion of phosphate into the urine, followed injection of parathyroid extract. Similar results were obtained in man by Albright and others.[10–12] According to Albright and Ellsworth,[7] the decrease in the serum inorganic phosphate level resulting from the heightened excretion of phosphate caused by parathyroid hormone tends to make body fluids less saturated with respect to calcium and phosphate ions and thus promotes *resorption* of bone salt and an elevation of the serum calcium. Following parathyroidectomy, according to this hypothesis, the sequence of events is similar, but the trends are just reversed. There is increased reabsorption of phosphate by the kidney resulting in a rise in serum inorganic phosphate. The calcium phosphate ion product is thus increased, favoring *deposition* of bone salt at the expense of the serum calcium.

According to these concepts concerning the mechanism of action of parathyroid hormone and the experiments which we have just described showing the extremely rapid decline in serum calcium following parathyroidectomy, it followed that an even more rapid rise in serum inorganic phosphate must occur. In preliminary experiments, the serum inorganic phosphate was determined in rats which had been parathyroidectomized four hours previously. The expected rise was not found, and we were thus led to investigate the question more intensively. A typical experiment is shown in FIGURE 3. Rats were kept on the low-calcium diet for 15 days, then parathyroidectomized. Blood was drawn from parallel groups of rats at several different subsequent times, and the serum was analyzed for calcium by the method of Fiske and Logan,[5] and for inorganic phosphorus by a modification of the method of Fiske and Subbarow.[13] It will be noted that the serum inorganic phosphate of the rats at zero time was much higher than that of normal human subjects. This higher phosphate is, in part, a characteristic of the species (the normal value for the adult rat is about 7.7 mg. per cent.[13]) The rats in this experiment were young, about 50 to 60 days old and, in common with human children,[14] young rats normally have higher serum concentrations of inorganic phosphate than adults.[15] Furthermore, low-calcium diets promote the more efficient absorption of dietary phosphate[16] (the experimental diet contained a normal amount of phosphorus), and this response was reflected in a higher concentration in the serum. According to our preconceived notion based on the Albright hypothesis, the serum inorganic phosphate should have risen prior to or coincident with the fall in serum calcium. But exactly the contrary result was observed; the serum inorganic phosphate fell at the same time as the serum calcium. We felt justified in making the obvious inference that, by parathyroidectomy and removal of the source of parathyroid hormone, there had been withdrawn the supply of an agent which acts directly to promote the dissolution of bone salt or to restrain its deposition or both. The parathyroids, therefore, appear to regulate the equilibrium of calcium and phosphorus between blood and bone in some primary and direct manner. True, some hours after the serum calcium had fallen precipitously, after the rats had gone into severe tetany and were in the terminal condition, the serum inorganic

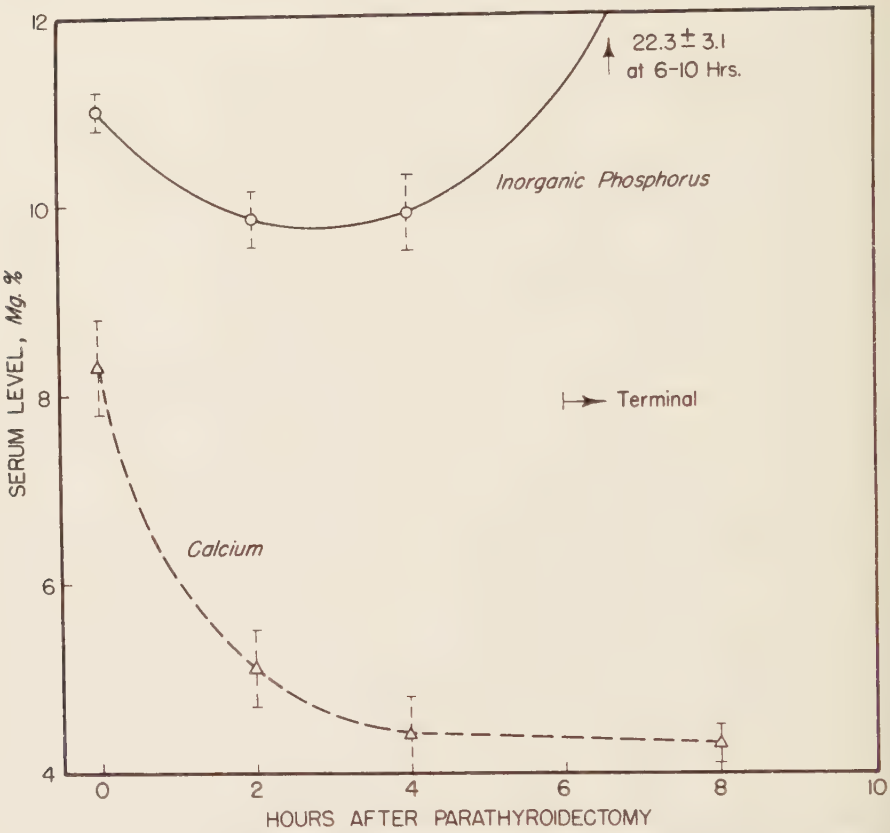

FIGURE 3. Serum calcium and inorganic phosphorus of calcium-depleted rats at varying time intervals after parathyroidectomy. The 8-hour point represents animals in severe tetany from 6 to 10 hours after parathyroidectomy.

phosphate soared to abnormal heights. Certainly, this change occurred tardily as compared with the fall in serum calcium.

Similar results have been reported by others in animals after parathyroidectomy[17] and in human patients following removal of parathyroid tumors.[18] Our experimental animals, although they exhibited no hypercalcemia, may perhaps have been in a state of hyperparathyroidism at the time of parathyroidectomy. Would the same sequence of events have been observed in rats initially euparathyroid?

In order to answer this question, an experiment was conducted on rats which had been fed a normal diet. A number of male rats of the same age and weight range were assembled. Half were fed the low-calcium diet for 17 days beginning at the age of 35 days; the other half were fed the same diet except that a liberal amount of calcium had been added. At the age of 52 days, they were parathyroidectomized. Two hours later they were bled for calcium and phosphate analyses. The results are shown in FIGURE 4. Our previous experiments on calcium-depleted rats were confirmed. There was a prompt fall in the serum

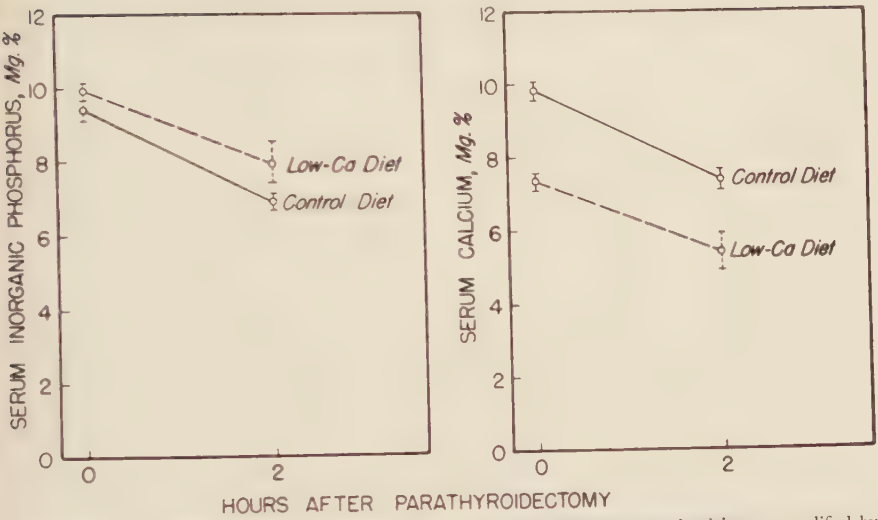

FIGURE 4. The effect of parathyroidectomy on serum inorganic phosphorus and calcium, as modified by dietary calcium intake prior to the operation.

inorganic phosphate as well as in the calcium. Furthermore, the rats on a normal diet responded to parathyroidectomy in a similar manner. Although the initial serum calcium of the rats on the normal diet was higher than that of the calcium-depleted rats, the magnitude of the fall was the same. The initial serum inorganic phosphate of the rats on the normal diet was slightly lower but it still fell about the same amount as that of the rats on the low-calcium diet. These experiments, therefore, show that, in euparathyroid as well as in hyperparathyroid rats, the fall in serum calcium following parathyroidectomy is not dependent on a prior rise in serum inorganic phosphate.

Additional evidence that the parathyroids have a regulatory effect on blood calcium independent of the effect on reabsorption of inorganic phosphate by way of the renal tubule was furnished by an experiment in which parathyroid extract was injected subcutaneously immediately following parathyroidectomy. Calcium-depleted rats were parathyroidectomized and immediately thereafter injected with 100 U.S.P. units of a commercial parathyroid extract per 100 gm. body weight. The serum calcium and inorganic phosphate were determined at three subsequent times, 2 hours, 6 hours, and 18 hours after parathyroidectomy (FIGURE 5). The initial drop in serum calcium was found to be less than that of similar rats not injected with extract (see FIGURE 1). Nevertheless, the fact that there was a fall observed at this interval indicates that absorption of hormone in the form and by the route administered had been delayed. The fall in serum inorganic phosphate was no different from that previously found at this time interval following parathyroidectomy in similar rats not injected with extract (FIGURE 3). In the experiment under consideration (FIGURE 5), therefore, it was not caused by the injection of parathyroid extract. At the end of six hours, the serum calcium had risen to the preoperative level, thus revealing the full effectiveness of the treatment. This rise was not accompanied by a

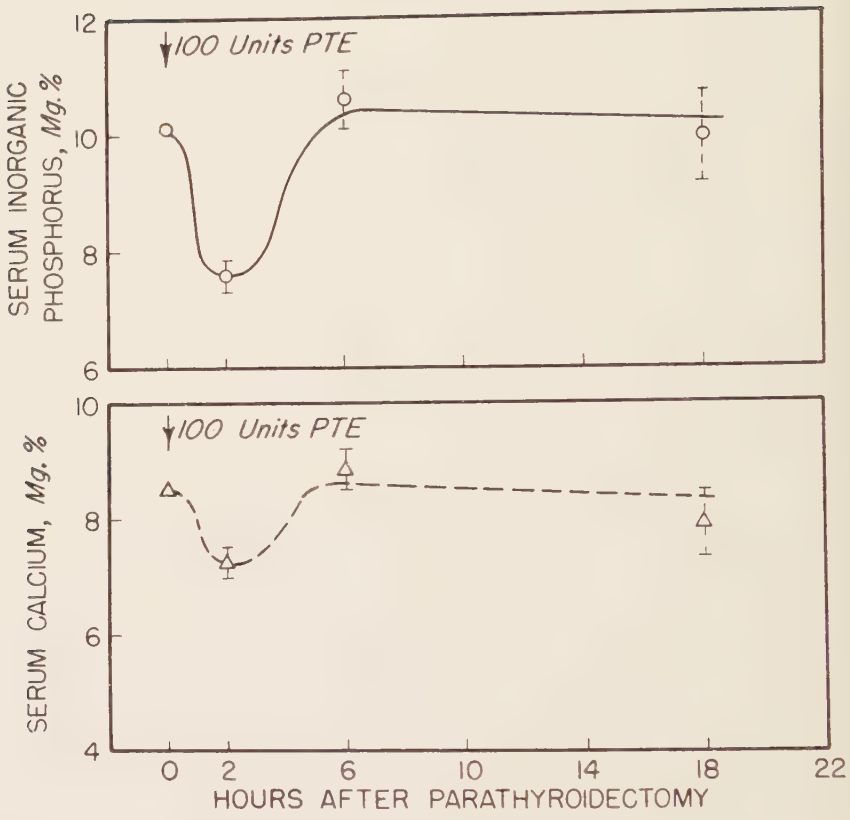

FIGURE 5. Serum calcium and inorganic phosphorus at varying time intervals after parathyroidectomy and injection of parathyroid extract (PTE).

further depression in serum inorganic phosphate. Instead, the level of this ion accompanied the calcium in its rise to the preoperative level. Twelve hours later, at the 18-hour interval, both the calcium and inorganic phosphate levels remained essentially unchanged.

We believe that the results just presented (which confirm earlier observations in intact dogs by Collip *et al.*[19] and Logan[20]) are best explained on the basis of a direct effect of the parathyroids on skeletal tissue independent of whatever effect they may have on the kidney tubule. We therefore suggest that the original hypothesis of Albright and Ellsworth[7] is inadequate to explain the mode of action of the parathyroids in the maintenance of a normal serum calcium. Writing in 1948, Albright and Reifenstein[15] also have stated: " . . . parathyroid hormone may act primarily on phosphorus metabolism in some way which not only increases the excretion of phosphorus in the urine but also produces certain bone changes directly." It is only proper to add that, in our discussions with Doctor Albright, he has indicated his continued belief that the effects on phosphate metabolism are of primary importance, while we are inclined to emphasize the effects on bone as more important.

Other workers have also furnished evidence that the regulatory effect of the parathyroids on the level of serum calcium is independent of the effect on the kidney: (a) parathyroid extract has been shown by histological criteria to stimulate osteoclastic activity and demineralization of bone;[21–25] (b) the serum calcium can be increased in nephrectomized animals by parathyroid extract, and recent investigators[26–29] are in complete agreement on this point (the earlier more controversial literature has been reviewed by Greep[6] and by Albright and Reifenstein[18]); (c) a specific local "solubilizing" action of parathyroid implants on bone has been demonstrated;[30, 31] (d) a differential inactivation of the calcium-mobilizing activity of parathyroid extract, with retention of the phosphaturic activity, has been claimed;[27] (e) the low phosphaturic activity of currently available commercial parathyroid extracts in the Ellsworth-Howard test[12] has been noted by several clinical investigators.[32–34]

If the evidence to date justifies our assumption that the parathyroids have at least two activities, one directly on bone and the other directly on the kidney, and that the two are in large part independent of one another, then there are two major alternative means by which these activities may be exerted: (a) there may be a single parathyroid hormone which possesses both activities; or (b) there may be two separate parathyroid hormones with distinct physiological properties (the second possibility has already been suggested by Talbot *et al.*[14] and some preliminary evidence in favor of it has recently been reported by Davies and Gordon[35]).

In order to investigate these alternatives, it appeared necessary to study the purification of parathyroid hormonal extracts and, as a first step in this direction, it was important to develop new biological assay techniques. We first directed our attention toward a method for assaying the calcium-mobilizing activity.

The official method of assay[36] for parathyroid extract depends upon the rise in serum calcium induced in intact dogs by a single subcutaneous injection of parathyroid extract. The U.S.P. unit is defined as 0.01 of the amount necessary to raise the serum calcium 1 mg. per cent, 18 hours after injection. This assay method apparently is a very satisfactory one for many purposes. Our facilities, however, do not permit the care and maintenance of an adequately large colony of assay dogs, and we hoped to develop a method in rats which would be more convenient for us, more economical, and more sensitive.

The intact rat is notoriously insensitive to parathyroid extracts. A relatively high dosage is necessary to cause a clear-cut increase in serum calcium.[37] We found, however, that a relatively small amount of extract injected subcutaneously into parathyroidectomized rats immediately following the operation caused a definite degree of maintenance of serum calcium which was proportional to the logarithm of the dose.

In order to establish optimum conditions for the new assay method, several factors were investigated further. The influence of the extent of calcium depletion was studied by maintaining rats on the low-calcium diet for varying periods of time immediately prior to parathyroidectomy. In the experiment shown in FIGURE 6, rats of uniform age were placed on the low-calcium diet 4, 9, or 12 days before parathyroidectomy, or not at all. They were then all

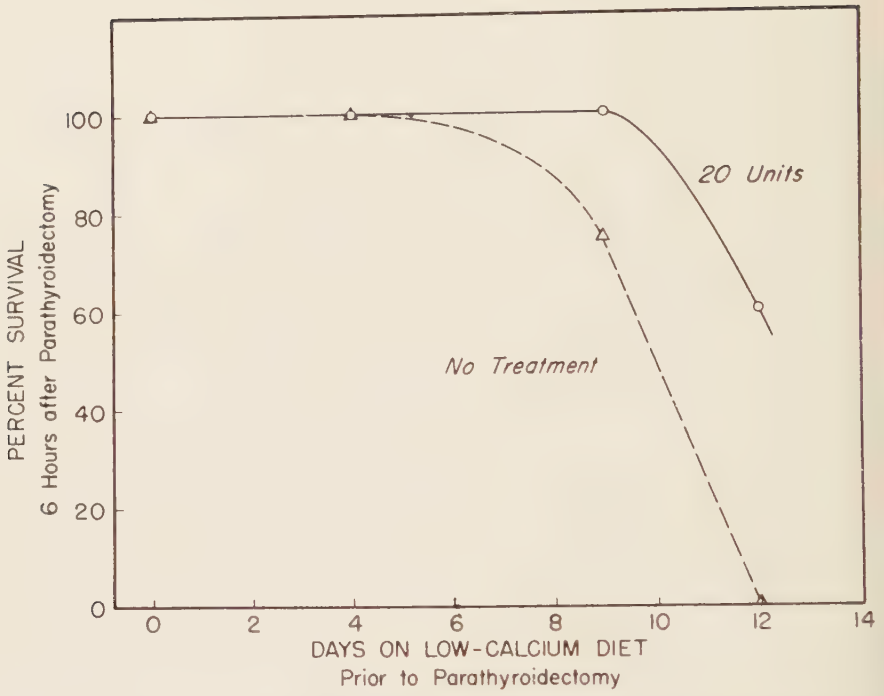

FIGURE 6. Effect of period of calcium depletion on 6-hour survival rate after parathyroidectomy, as influenced by injection of 20 U.S.P. units of parathyroid extract (per 100 gm. body weight) immediately following parathyroidectomy.

parathyroidectomized on the same day at the same age and kept without access to calcium for the next six hours, when they were bled for calcium analysis Half of these rats were untreated, the other half were injected subcutaneously, immediately after parathyroidectomy, with 20 U.S.P. units of commercial parathyroid extract per 100 gm. body weight. The effect on survival is shown in FIGURE 6. The 12-day depletion period was too long. All the untreated rats and 40 per cent of the injected rats died within the six-hour interval. In this series of rats even a nine-day depletion period resulted in some mortality. When the rats were depleted of calcium for only four days, however, all the rats survived parathyroidectomy for six hours, even when no treatment was administered, and the difference between treated and untreated rats was the most marked of any of the groups. The serum calcium values from the same experiment are shown in FIGURE 7. Although administration of 20 units of extract increased the mean survival time of rats depleted of calcium for 12 days (FIGURE 7), the mean serum calcium level of the survivors at six hours was extremely low. It may also be seen that only a slight betterment in serum calcium over the controls resulted from treatment of the nine-day group with parathyroid extract. When the rats were depleted of calcium for only four days, however, the difference between treated and untreated rats was the most marked of any of the groups. Although the group of rats not depleted of calcium at all showed the

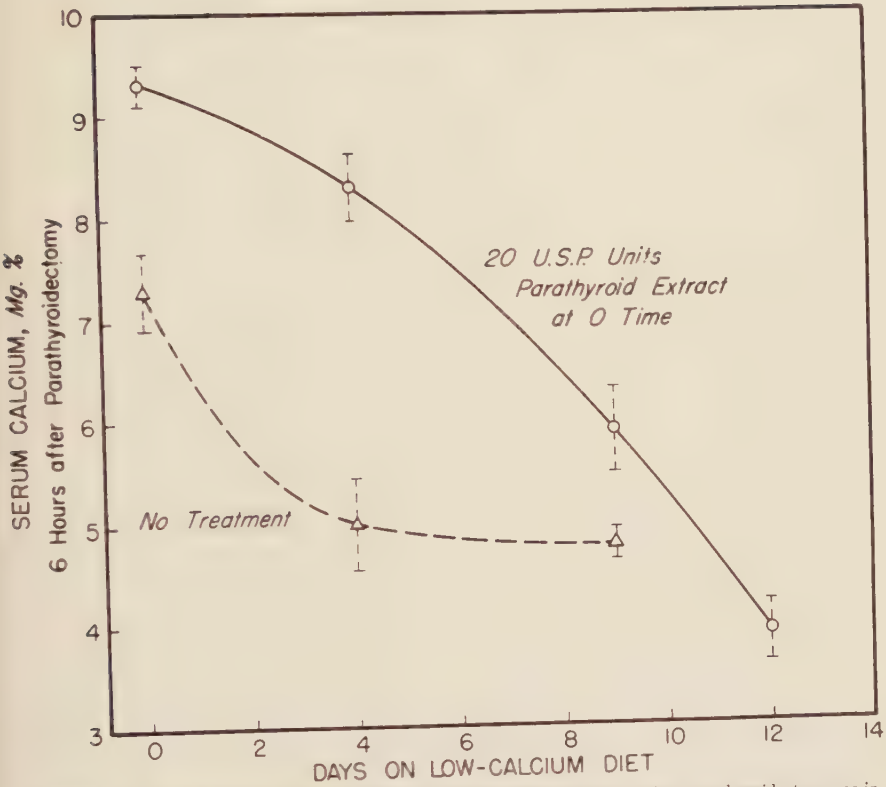

FIGURE 7. Effect of period of calcium depletion on serum calcium six hours after parathyroidectomy, as influenced by injection of 20 U.S.P. units of parathyroid extract (per 100 gm. body weight) immediately following parathyroidectomy.

highest serum calcium under treatment, the increment above that of the untreated group was relatively small. On the basis of this and other experiments, the four-day calcium-depletion period was selected as most advantageous for the biological assay method. Rats thus treated appeared to be most sensitive to small doses of extract and, in addition, a reasonable span of response was made available for evaluating extracts with varying potencies. Subsequently, in another experiment, depletion periods of two, four, and six days were compared. No great differences in values were observed, but, if anything, the four-day period appeared to be most satisfactory.

The most advantageous time interval between parathyroidectomy and withdrawal of blood sample for calcium analysis was also studied. Two-, four-, and six-hour intervals were selected for test as shown in FIGURE 8. Different doses of parathyroid extract were administered, 5, 20, or 80 U.S.P. units, respectively, of commercial extract, per 100 gm., to each group. The variability(s), slope of the log dosage-response curve (b), and index of precision (λ) were calculated, and the time interval originally selected (six hours) appeared to be the best. The low variability in the two-hour group was noteworthy, but this advantage was

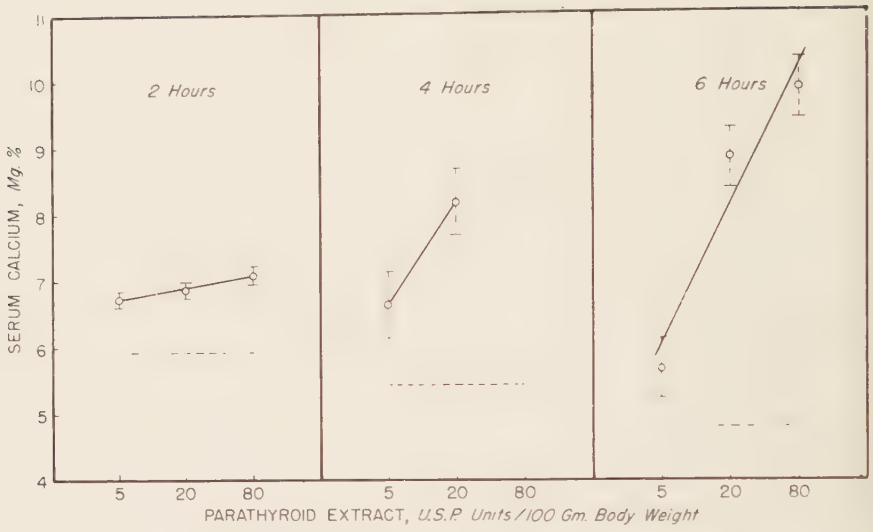

FIGURE 8. Effect of time interval between parathyroidectomy and blood collection on character of log-dosage response curve for parathyroid extract injected immediately after parathyroidectomy. The horizontal dotted lines represent the mean serum calcium values for untreated rats at the indicated intervals.

more than cancelled out by the low slope. Nevertheless, additional experiments were conducted in the hope that the low slope observed here had been a chance occurrence. Such was not the case, however, and, at present, it appears that the six-hour interval is best for the assay in parathyroidectomized rats maintained on a low-calcium diet for four days before parathyroidectomy.

Varying times of administration of extract were also tested, but injection immediately after parathyroidectomy was found to be preferable.

As a result of these experiments, the specifications for the biological assay method were set as follows.[38]

Male rats of the Sprague-Dawley strain are obtained from the Holtzman Rat Co., at about 40 to 45 days of age, and are kept on a stock diet of laboratory chow until 4 days before the assay. They are then placed on the calcium-deficient diet of Shaw[4] and four days later, are parathyroidectomized by cauterization by the method of Greep. They have previously been randomized into groups of five or six according to body weight. Immediately after the operation, two groups of rats are injected with 5 and 20 U.S.P. units, respectively, of Lilly Parathyroid Extract per 100 gm. body weight (or equivalent doses of a house standard). Other groups of rats are injected with varying aliquots of experimental extracts of unknown potency. In tribute to the reliability of the official U.S.P. method of bioassay and to the high standards of the Lilly Co., we have not found significant differences in potency between different batches of the Lilly extract. Exactly six hours after operation and injection, the rats are anesthetized with ether and bled by heart puncture. The blood samples are centrifuged and the serum is withdrawn. Originally, the calcium content was analyzed by the classical method of Fiske and Logan,[5] but more recently we have utilized a new unpublished method developed by Iseri and Munson, based

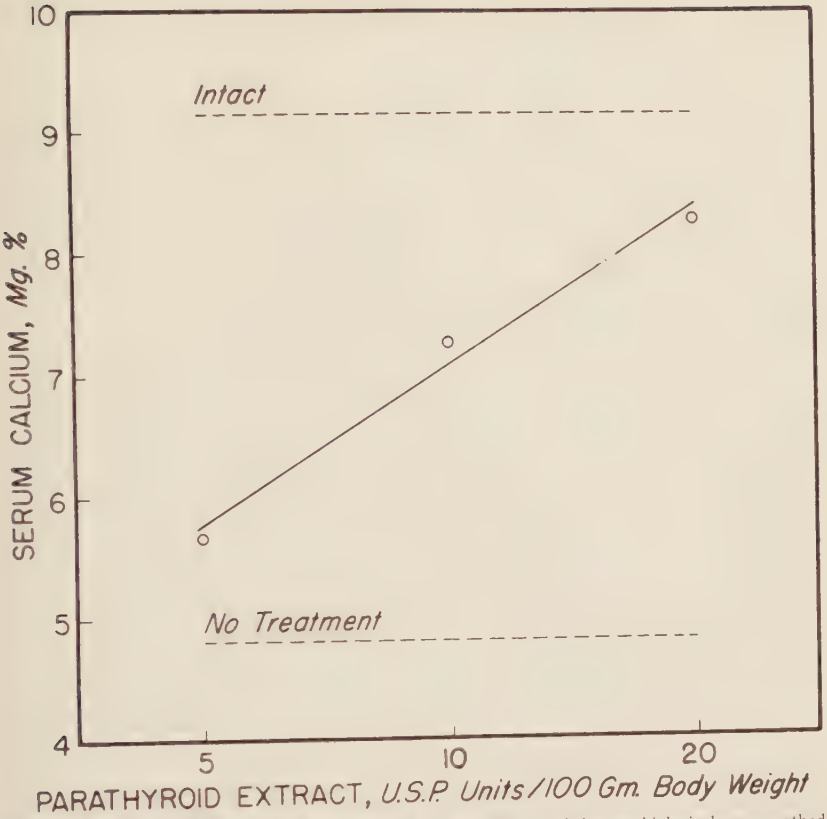

FIGURE 9. Log dosage response curve obtained in the first 15 trials of the new biological assay method for the calcium mobilizing activity of parathyroid extract. The horizontal dashed lines represent the mean serum calcium values of intact rats, and of parathyroidectomized rats six hours after operation. All rats were maintained on the same dietary regime.

on titration with ethylenediamine tetraacetate in a spectrophotometer. The new method requires only 0.5 ml. or less of serum, and takes only two to five minutes for completion. Since the development of the new bioassay method, 50 assays, each on approximately 30 rats (a total of over 1400 rats), have been conducted according to these specifications. FIGURE 9 summarizes the results obtained with Iseri and Doctor Kenny on the first 15 assays. Subsequent assays have given comparable results. The mean index of precision (λ) is about 0.27, which means that, if two doses of standard are compared with two doses of unknown, with five rats in each group, and a good match is obtained, then the potency will be estimated with a standard error of about ± 30 per cent. It is still hoped that we may be able to devise modifications of the assay method which will result in a further improvement in precision, but, as it stands, it is reasonably satisfactory in this regard, and has a special appeal on the basis of convenience, economy, rapidity, sensitivity, and specificity. Other methods recently developed for the bioassay of calcium-mobilizing activity, using intact or parathyroidectomized rats,[39–41] have been reviewed by Greep and Kenny.[2]

Our effort was now directed toward development of an assay method for the phosphaturic activity of parathyroid extracts, since the methods previously described[42, 43] were not sufficiently precise for our purpose. The early experiments were carried out with Doctor B. G. Vine and have been carried further with Doctor A. D. Kenny. In confirmation of previous workers, it was first found that parathyroidectomy in rats results in a decrease in the urinary excretion of inorganic phosphate whether measured for a 6-, 12-, or 24-hour period immediately following the operation. When parathyroid extract is administered either to intact or to parathyroidectomized rats, there is an increase in urinary inorganic phosphate. However, the response of the parathyroidectomized rats is more uniform and is manifested in a clear-cut manner with a smaller dose of extract. An additional reason for preferring parathyroidectomized rats for the biological assay method is that the lower baseline of control untreated parathyroidectomized rats makes available a larger range of responses for testing varying doses and potencies. At first, it was thought that the rats, depleted of calcium for four days and used in the bioassay method for calcium-mobilizing activity, could be used simultaneously for the phosphaturic assay. Under this scheme, these rats would be put into metabolism cages immediately following parathyroidectomy and injection with extract, and urine would be collected during the six-hour waiting period between injection and bleeding. This possibility is still being actively investigated, but results to date indicate that a higher degree of precision is possible if rats used in the phosphaturic assay are maintained on a normal stock diet throughout the period immediately prior to parathyroidectomy. Actually, there is not always any material advantage in the use of the same rats for both assays, because it is often desired to test different extracts or different dosage levels by one or the other of the two methods.

As a result of the experiments just noted, specifications for the phosphaturic assay method have been selected as follows.[44] Male albino rats obtained from the Holtzman Co., are maintained on a stock diet of laboratory chow. At a uniform age of 50 to 60 days, they are parathyroidectomized by cauterization and, immediately thereafter, are injected subcutaneously with a low (0.2 ml.) or high (0.8 ml.) dose of a crude parathyroid extract prepared by Doctor Kenny. This extract at present is serving as our house standard. The rats are then immediately put into individual metabolism cages with access to water but not to food. The urine voluntarily excreted by each rat and the cage rinsings are collected for the following six hours, and suitable aliquots are analyzed for inorganic phosphorus by the method of Fiske and Subbarow.[13] In order to keep the procedure as simple and convenient as possible, no effort is made to empty the bladder either at the beginning or end of the urine collection period, nor have gavages been employed. In one experiment, creatinine as well as phosphate analyses were carried out, but there was no indication of a helpful correlation between the two determinations. Selection of a six-hour urine collection period, rather than one of shorter duration, is probably responsible for the apparently minor importance of imperfections in the technique of urine collection.

The combined results of the first five phosphaturic assays conducted according to the specifications outlined are shown in FIGURE 10.

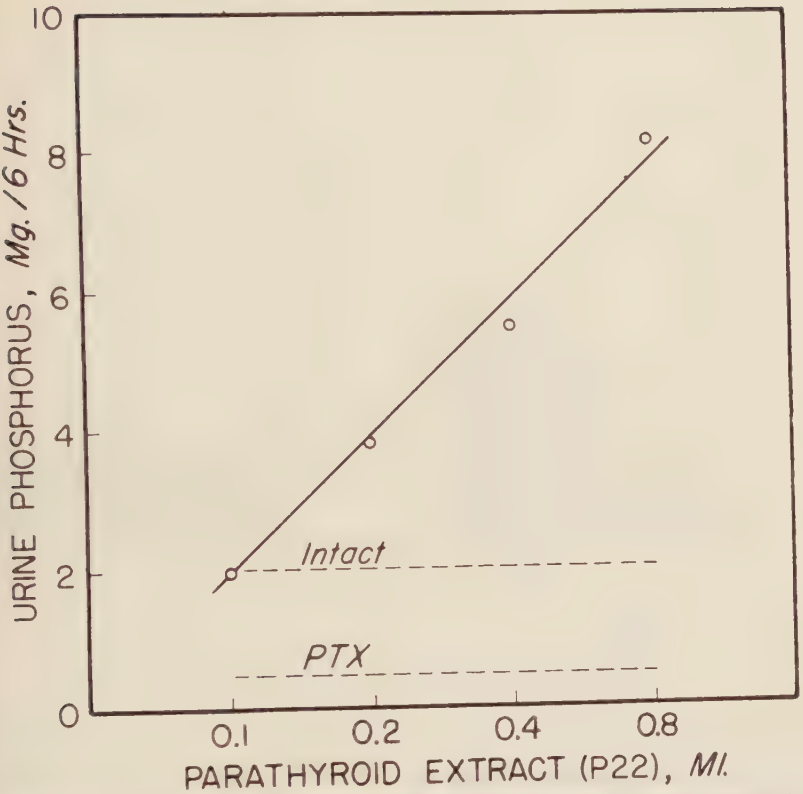

FIGURE 10. Log-dosage response curve obtained in the first five trials of the new biological assay method for the phosphaturic activity of parathyroid extract. The horizontal dashed lines represent the mean values for urinary inorganic phosphate by parathyroidectomized rats during the six hours immediately following parathyroidectomy and for intact rats during the same time interval. All rats were maintained on the same dietary regime.

The log-dosage response curve was satisfactorily linear when drawn to the best fit for the four dosage levels tested. The mean index of precision (λ) was 0.27, the same as for the calcium-mobilizing assay method. The mean values for untreated parathyroidectomized rats, and for otherwise comparable untreated intact rats, are also shown in FIGURE 10. It is interesting that the level of phosphaturia was easily increased above the control value for intact rats by small doses of parathyroid extract. In contrast, very large doses would be required in order to elevate the serum calcium above the intact control value.

It was mentioned earlier without detailed comment that, in recent years, some clinical investigators have experienced difficulty in carrying out the Ellsworth-Howard test successfully. In FIGURE 11, the recent results of Dent, Kenny, and Philpot working at University Hospital, London[45] with this test are contrasted with the results obtained ten years earlier by Albright, Burnett, Smith, and Parson.[46] We understand that the results of Dent and his colleagues are typical of the recent experience of other workers, although there are exceptions to this statement. One may note that, when normal subjects were

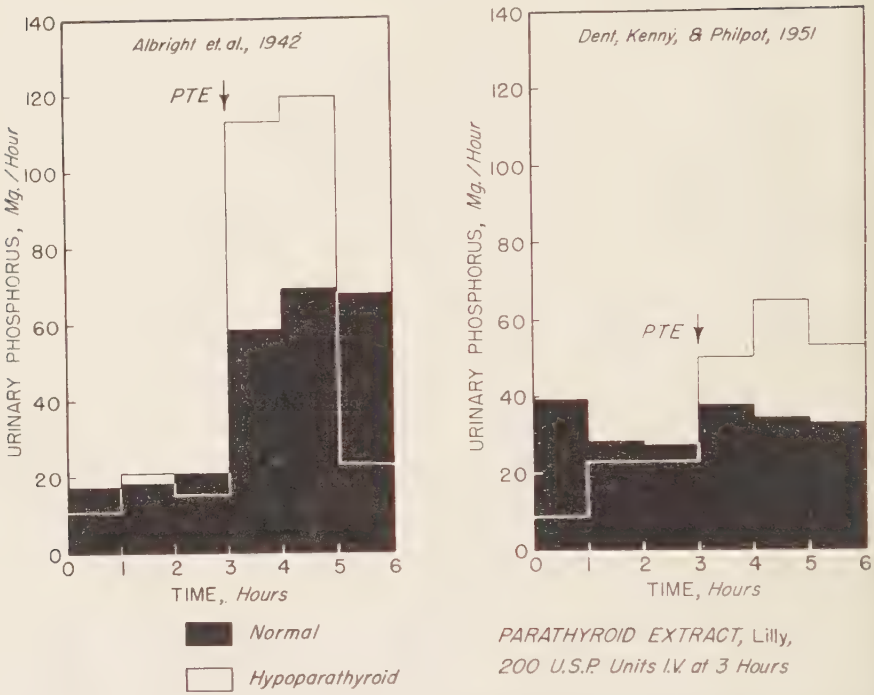

FIGURE 11. The phosphaturic effect of parathyroid extract in normal and hypoparathyroid human subjects observed by Dent, Kenny, and Philpot in 1951, as compared with the effect reported by Albright *et al.*[45] in 1942.

administered 200 U.S.P. units of currently available commercial extract (Lilly), no significant increase in phosphate excretion occurred, and even the response of hypoparathyroid patients was minimal. In contrast, the test as carried out a decade ago resulted in a tremendous increase in phosphate excretion by the hypoparathyroid individual, and the normal subject also responded with a highly significant increase. It occurred to us that the discrepancy might be explained rationally on the basis of two assumptions: (a) parathyroid extract contains two separable hormonal activities, one independently calcium-mobilizing and the other independently phosphaturic in its action; (b) over a 10-year period the manufacturing process for parathyroid extract has been changed in such a way as to retain the previous level of calcium-mobilizing activity but, in the process, the phosphaturic activity was lost in part. Our own experiments certainly provide no indication that the calcium-mobilizing activity of present-day extracts in rats is any lower than that reported by earlier investigators who used the older extracts.

To investigate this proposed explanation and to further our study of the possibility that there are two separable parathyroid hormones, we compared the phosphaturic activity of our own crude parathyroid extract with the partially purified commercial extract. First, the number of units of calcium-mobilizing activity per milliliter in our crude extract was determined by bioassaying it against a commercial extract which had previously been reliably assayed in dogs

by the official U.S.P. assay method[36] and contained 100 U.S.P. units per milliliter. In each of two assays,[38] two dosage levels of each extract were administered to groups of five or six rats each, and the potency and standard error of estimate were then calculated by standard statistical techniques.[47] The two assays were in reasonable agreement, and the weighted mean potency was 35.8 ± 8.1 "calcium" units per milliliter for the crude extract.

The same two extracts were now tested in parallel by our bioassay method for phosphaturic activity.[39] Three assays were carried out in which two dosage levels of each extract were administered to rats. The dosage levels for both extracts were 5 calcium units and 20 calcium units. For the Lilly extract, they were 0.05 and 0.2 ml., respectively; for the crude extract they were 0.15 and 0.60 ml. All three assays showed the crude extract to be more potent as a phosphaturic agent. The three values obtained were 3.9, 5.1, and 2.7, respectively, and the weighted mean was 3.6 ± 0.67. In other words, the phosphaturic potency of the crude extract was three to four times as great as the purified extract, when the two solutions were tested against each other on the basis of equal dosages of "calcium" units.

We have some evidence that high phosphaturic activity is not a peculiar property of crude extracts. A somewhat purified extract prepared by Doctor Kenny, which has twice the specific activity of the crude extract in terms of calcium-mobilizing activity, appears to have suffered no loss of phosphaturic activity in the process of concentration.

These findings have provided us with some evidence that the calcium-mobilizing activity and phosphaturic activity of parathyroid extracts can be partially separated, and there would seem to be reason to believe that two separate hormones may possibly exist in the parathyroid gland. Current difficulties with the Ellsworth-Howard test might be explained on this basis. With the aid of the two new biological assay methods which we have described, we are encouraged to undertake additional efforts to separate the two hormonal activities to a greater extent and to purify them, if that is possible.

The concept that the parathyroid may elaborate more than one hormone is not original with us for it has been suggested by several authors. Indeed, Doctor Philip Handler and his colleagues[45] have reported data which suggest the presence of four pharmacologically active constituents of parathyroid extract.

There is still another function of the parathyroids which has received little attention and which we believe deserves further study. It has been known, for some time, that thyroparathyroidectomy of lactating animals results in a diminution in the rate of milk production.[49] More recently Cowie and Folley,[50] in experiments with rats, established the fact that the parathyroids and not the thyroid are responsible. Experiments in our laboratory with Doctor Svein U. Toverud and Doctor Alexander D. Kenny[51] are confirmatory and add some additional information.

The study was initially undertaken in search of a method for producing hypocalcification of the molar teeth of the rat as a preliminary to a study of the effect of this condition on caries susceptibility. Molar calcification in the rat, for the most part, takes place during the suckling period,[52] and it was reasoned that, in order conveniently to affect this process deleteriously, it would be nec-

essary to reduce the calcium intake through the mother's milk. Two possible methods were considered because of their known production of hypocalcemia, restriction of the dietary intake of calcium by the mother, and parathyroidectomy.

Rats from Holtzman stock which had been bred and maintained during their first pregnancy in our laboratory were then studied during the ensuing lactation period (we are indebted to Doctor Ernest Knobil for supervision of this phase of the investigation). In the first experiment, the litter size was reduced to six uniformly, and all lactating rats were maintained on a stock diet of laboratory chow until the 11th day of lactation. Eleven of the rats were then changed to a low-calcium diet of Shaw,[4] and milk samples were obtained on the 13th, 15th, and 19th days of lactation. Six rats were kept on a stock diet, and similar milk samples were obtained from them. Three rats on the stock diet were parathyroidectomized on the 13th day of lactation, and milk was obtained on the 15th and 19th days. The mean calcium concentration of the milk for the various groups is shown in FIGURE 12. We were immediately impressed with the constancy of this determination. The close agreement between the mean values for the normal group from one day to another and the small standard errors may be noted. This constancy encourages us to believe that it is feasible to study this particular aspect of the lactation process in the rat without the difficulty of excessive animal variability. The same uniformity was observed in the group on a low-calcium diet; a marked (30 per cent) decrease in milk cal-

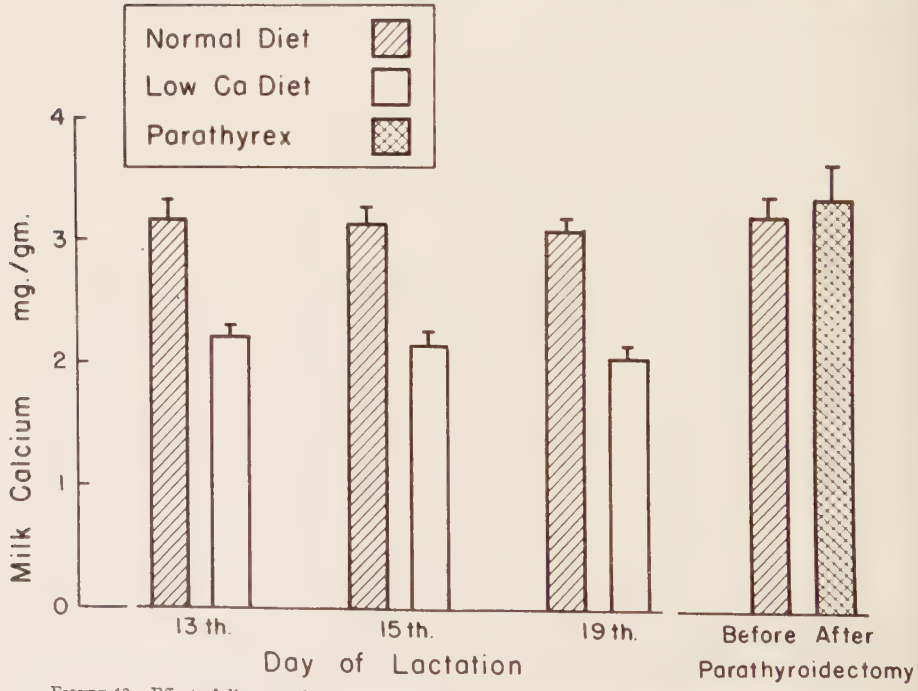

FIGURE 12. Effect of dietary calcium intake and of parathyroidectomy on the calcium concentration of rat milk (first experiment).

cium concentration following the imposition of the low-calcium diet. We had hardly expected that any decrease would become evident so soon after imposition of the dietary insult, that is, in two days. It was also remarkable that, once a 30 per cent decrease had occurred, no further significant decline in milk calcium concentration was observed. The milk calcium concentration of the rat is very high, about 10 times that of the human female, for example,[53] and, even during the observed decline, thus remained seven times that of the human.

Blood samples were also collected from these animals, but unfortunately they were lost before the analyses could be completed. Nevertheless, we were reasonably certain that, on a low-calcium diet, the lactating rat would show a decline in blood calcium which, although small, would be sufficient to stimulate the parathyroids to hyperactivity. The possibility that the parathyroids were stimulating the mammary glands just as they do the skeletal tissue, in the interest of the maintenance of blood calcium, was further supported by observations on the parathyroidectomized lactating rats. If the level of serum calcium alone were responsible for the reduction in milk calcium concentration and were correlated with it, then, surely, parathyroidectomy would result in an even further decline in milk calcium. Such a decline did not appear to be the case in this experiment. Although the values charted for parathyroidectomized rats are each based on milk samples from only two of the three rats in this group, it is clear that no significant reduction in milk calcium concentration occurred in this group in spite of an almost certain hypocalcemia.

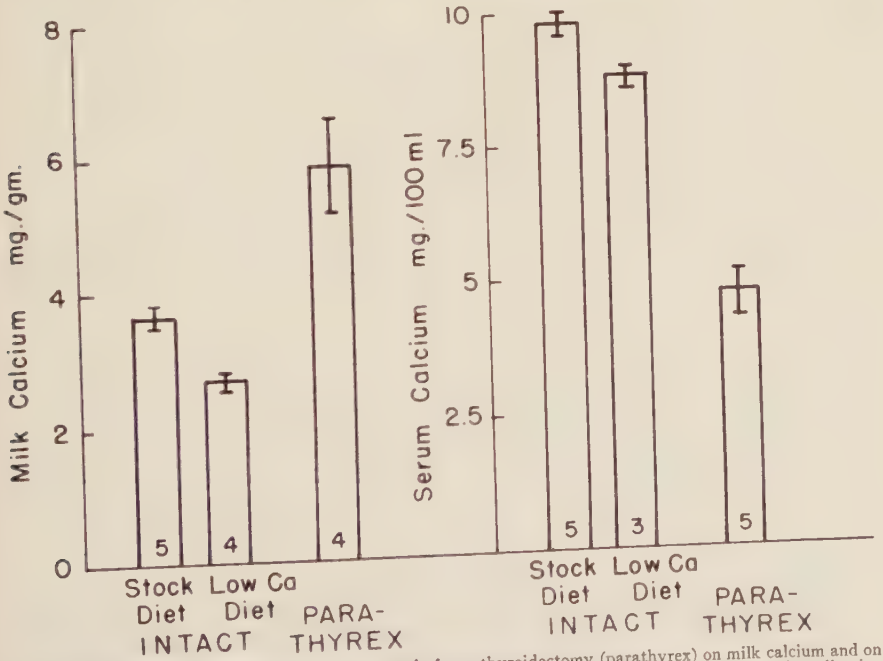

FIGURE 13. Effect of dietary calcium intake and of parathyroidectomy (parathyrex) on milk calcium and on serum calcium in rats (second experiment). The blood samples were drawn immediately following the collection of milk. The number of rats in each group is indicated on the corresponding bar.

A second experiment primarily for the purpose of obtaining values for serum calcium as well as milk calcium, and for extending the observations on parathyroidectomy was then conducted, and the results are shown in FIGURE 13. As expected, the mean serum calcium of the intact rats on the low-calcium diet was somewhat lower (about 1 mg. per cent), and that of the parathyroidectomized rats was very much lower than normal. In this experiment, again, the low-calcium diet was imposed on the 11th day of lactation and the milk samples were collected two days later. In those rats in which parathyroidectomy was performed, the operation was carried out on the 11th day of lactation. In confirmation of the first experiment, a reduction of 30 per cent in milk calcium concentration was again observed two days following imposition of a low-calcium diet. The trend toward an increased milk calcium concentration of the parathyroidectomized rats, only dimly discernible in the first experiment, became very marked in the second experiment, two days after parathyroidectomy. Although, several days later, in values not charted, there was a return of milk calcium to control levels, no reduction below the controls was observed.

We are in no haste to postulate a third parathyroid hormone to account for the effects on the process of lactation. But we are interested in investigating this activity further and in correlating it with the effects on bone and kidney.

Summary

We have presented new evidence to support the concept that the parathyroids act to maintain the blood calcium level by a mechanism independent of the kidney, presumably directly on the bone. We have described two new biological assay methods based on the responses of parathyroidectomized rats, the one for the calcium-mobilizing activity, and the other for the phosphaturic activity of parathyroid extracts. With the aid of these two assay methods, we have presented evidence that it is possible to separate partially the two hormonal activities. Further strength has been lent to the idea that the parathyroid may contain and secrete more than one distinct hormone. Finally, we have reported data which suggest that the parathyroids may influence the calcium-concentrating mechanism of the mammary glands, as well as their over-all metabolism.

Acknowledgments

In conclusion, I wish to express my gratitude to Doctor Roy O. Greep, my collaborator in much of the work reported here, for guiding me into this most interesting field of research. I also wish to acknowledge the able collaboration of Oscar A. Iseri and of Doctors Alexander D. Kenny, Svein U. Toverud, and Bernard G. Vine.

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Discussion of the Paper

Doctor Isidor Greenwald (*New York University College of Medicine, New York, N.Y.*): It is interesting to have Doctor Munson confirm, on the basis of experiments made on the rat, the results we obtained many years ago experimenting with dogs, namely that, after parathyroidectomy, the fall in the con-

centration of calcium in the serum may be accompanied by but little or or no increase in the concentration of phosphate. Doctor Munson also seems to have confirmed the results of Collip, Clark, and Scott, who found that the increase in the concentration of calcium in the serum after the administration of parathyroid extract was not accompanied by a decrease in the concentration of phosphate. Nevertheless, I do not believe that it is necessary to assume that there are two active substances in parathyroid extracts in order to reconcile Albright's results with those that indicate that the action of the parathyroid is not upon the kidney but upon the bone. It seems to me that Albright erred in assuming that the earliest *detected* change was actually the primary and only *direct* effect of the presence, or absence, of the parathyroid hormone. It may well be that the human kidney responds to small changes in the concentration of calcium, changes too small to be detected in the ordinary method of analysis with an increase or decrease in the excretion of phosphate. Perhaps the response is to some other product of the action of the hormone, for it is possible that the hormone is not, itself, the agent that acts upon bone.

PANEL DISCUSSION

Moderator: Albert E. Sobel

Doctor A. Baird Hastings (*Harvard Medical School, Boston, Mass.*) The papers by Doctors Hendricks, Hodge, Armstrong, Neuman, and Traute have formed a group, all dealing with the inorganic portion of bone, dentin, and enamel. As distinguished from other papers in this monograph, they have not dealt with the organic matrix. Instead, naturally, they have discussed crystal structure, crystal growth, and the physical chemistry of two-phase systems. Put in these general terms, one might incorrectly be led to the conclusion that the topics discussed in these papers are a rehash of subjects under investigation for at least 25 years. But that would be far from the truth. In the first place, there have been powerful additions to our experimental tools since the early 1920's. In the second place, a number of different kinds of highly specialized experts have become interested in the problem of calcification (one might say that research on the bone salt has lost its amateur standing). Doctor Kramer, Doctor Holt, and Doctor Sendroy will remember how we severally and hopefully plunged into the problem of accounting for the formation and solution of bone salt, on the simple assumptions: (1) that chemical analysis would show what the bone salt was; (2) that it would have a characteristic solubility product; (3) that solutions (and plasma) could be brought into equilibrium with it; and (4) that analysis of plasma would reveal whether body fluids were or were not in equilibrium with the bone salt.

A great amount of careful and painstaking work resulted; and I am not as pessimistic about the value of the data obtained as I am about some of the conclusions drawn. For example, work carried out on the solubility of single batches of the solid phases, calcite, aragonite, and OH-apatite [then called $Ca_3(PO_4)_2$], gave reasonable changes in ion products with varying ionic strength, reproducible in individual laboratories. It is hard for me to account for this phenomenon without supposing that some sort of an equilibrium between the liquid and solid phases had been established. However, the question of whether body fluids are or are not in equilibrium with the bone salt was not answered by these earlier experiments.

Many things have happened in the past quarter of a century. We have gone through a series of revisions on what the bone salt is: the concept presented by Doctor Hendricks seems to be consistent with the facts and very convincing. It seems to me that growth of the crystal to some maximal size (limited by the $CO_3^=$ makes a lot of sense) though I don't see how it would help us account for the peculiar solubility characteristics of the bone salt. You can see that I am still impressed with reproducibility of the data obtained by the same people with the same batch of bone salt. If we must abandon the idea of a single solid phase, perhaps we should think of a repetitive surface which can reach an equilibrium with extracellular fluids.

As a result of the discussion, I have been left with the impression, either that the solid phase of bone is unknowable, or that equilibrium between the liquid and solid phases is unattainable. Personally, I am not so pessimistic, though my own data are hardly up-to-date enough to be used as evidence.

Though it is true we did not know what the chemical composition of the crystal surfaces were, our results were reproducible, and we got the impression that we were reaching an equilibrium.

I agree with Doctor Hodge that $CaHPO_4$ is first formed in solution, prior to its transformation to the tertiary calcium phosphate salt. This suggestion is only reasonable from physicochemical considerations. In other words, for the formation of apatite from artificial solutions, the solubility product of $CaHPO_4$ must be exceeded. This step will then be followed by conversion to $Ca_3(PO_4)_2$ and increased acidity of the overlying solution, a change which all who have worked with the system have long recognized.

On citrate. A point on which I should like to comment briefly has to do with the problem of *intracellular citrate*, the conditions leading to its formation and the consequences of its accumulation. I call your attention to the work carried out in Professor R. A. Peters' laboratory at Oxford on citrate accumulation in tissues as the result of inhibition of the enzyme aconitase by fluoro citrate (caused by condensation of fluoro acetate and oxalacetate). A further consequence might also be noted: wherever citrate accumulates locally, calcium, if available, will be accumulated in the form of slightly ionized calcium citrate molecules. Should a similar series of events occur in osteocytes, due to a relative decrease in aconitase activity from whatever cause, citrate might be expected to accumulate intracellularly and the activity of divalent ions reduced accordingly.

My point is that if this accumulation can occur in other tissues, why may it not also occur in calcifying tissue? If citrate could subsequently be disposed of by metabolic activity, or by diffusing away, it would leave the Ca stranded, permitting it to combine with the HPO_4 to form $Ca_3(PO_4)_2$. This is sheer speculation, but it interests me enough to put it to experimental investigation. We seem to have plenty of ways of providing phosphate for calcification, but calcium has always seemed to me to be in short supply.

On precipitation of $CaCO_3$. I have some ancient data to call to your attention. One of the experiments hidden among the 123 pages published 25 years ago by Doctor Sendroy and myself on the solubility of calcium salts is one in which we equilibrated blood serum with $Ca_3(PO_4)_2$ for varying short lengths of time (specifically five minutes, one hour, and three and one-half hours).

During this period, the serum PO_4 did not change, but the serum Ca and serum HCO_3 (corrected to the initial pH) did change markedly. In five minutes the Ca had dropped 2.45 mM and the HCO_3 4.6 mM; in one hour the Ca had dropped 2.57 mM and the HCO_3 5.6 mM; and in three and one-half hours, there was no further change.

The point that I want to recall is that, for each Ca^{++} which disappeared, two HCO_3^- ions disappeared at constant pH. Presumably, both were taken up by the solid phase $Ca_3(PO_4)_2$. The equivalence of the changes is too close to disregard.

My question is: did the calcium phosphate salt take up Ca + two HCO_3 ions—or was Ca + CO_3 taken up with the release of one H_2CO_3 molecule? This is a question which might be settled, I believe, by repeating the experiment using C^{14} labeling of HCO_3 ion.

General questions to be worked on:

(1) Since there is biological equilibrium between body fluids and bone, how is this equilibrium brought about, by chemical inevitability or by metabolic activity?

(2) Since bone salt grows by crystal growth from biological solutions of controlled concentrations of constituents, what are the local factors which insure the accumulation of the appropriate ions in the appropriate concentrations?

The Nature of the CO_2 in Bone

Doctor Harold C. Hodge (*University of Rochester, School of Medicine and Dentistry*): The hydroxylapatite lattice can be satisfactorily portrayed by a suitable arrangement of hydroxyl ions placed at the corners of the diamond-shaped unit cell, of phosphate tetrahedra arranged in a complex pattern of interlocking hexagons, and of calcium ions with associated oxygens regularly oriented with relation to the PO_4 groups. It is generally agreed that such a structure determines the space arrangement of the principal components in the mineral of enamel, dentin, cementum, and bone. In addition to the calcium, phosphate, and hydroxyl ions, the next most important constituent of the hard tissue mineral is carbon dioxide. These constituents make up a substantial percentage of the total mineral weight. The human bone, for example, contains four or five per cent of CO_2 (inorganic basis).

There have been two principal hypotheses to account for the presence of the CO_2 in bone. One of these has placed the CO_2 *in the crystal lattice*, the other has assumed that the carbonate is *on the surface* of the crystals. Proponents of the first hypothesis have often used the name "carbonate apatite" to describe the mineral. This designation created considerable misunderstanding because some assumed that a carbonate apatite would have carbonate replacements in the hydroxyl positions analogous to the fluoride replacement of hydroxyl in fluorapatite. Bale showed that it would be impossible to substitute a CO_3 group for an OH group without altering the lattice dimensions to such an extent that easily discernible shifts would occur in the positions of major lines in the X-ray diffraction pattern. The near identity of the X-ray diffraction patterns of bone mineral and of fluorapatite was conclusive evidence that the bone mineral was not a carbonate apatite in this sense, specifically that CO_3 does not enter the lattice position occupied by hydroxyl groups in hydroxylapatite. More recently, carbonate substitutions for phosphate in the lattice have been proposed by McConnell. He has described a crystal with unchanged lattice dimensions in which other ions have been simultaneously replaced; for example, calcium ions by uncharged water molecules, thus preserving proper electrostatic charge balances. At present, there has been no rigorous demonstration that such substitutions do, in fact, occur.

On the basis of recent studies both *in vitro* and *in vivo*, however, a strong case can be made for the presence of at least some of the CO_2 on the surface of the mineral crystals. That bone crystals have an extraordinarily large surface area is now well established. Direct inspection in the electron microscope of crystals freed from organic material by a number of procedures has shown the crystals to be extremely minute. Typical dimensions, for example, may be 30 by

100 by 150 Å. Even these extremely small crystals probably are not perfect. Numerous faults may have occurred in the lattice as the crystals grew, so that there were left a large number of irregular, imperfect areas, some in the interior of the crystal, and some available (albeit with more or less restriction) to the external fluid layer. This very large crystal surface offers the opportunity for adsorption of carbonate. In fact, the capacity for adsorbed CO_2 is sufficient to account for the total found in bone.

Partial solubility studies have shown that the solution obtained from the treatment of powdered bone with a small amount of acid, an amount insufficient to dissolve the entire sample, always contains much more CO_2 relative to calcium and phosphorus than is present in the original bone powder. If the CO_2 is surface bound, it would be easy to understand that it could be leached out preferentially.

Sobel and colleagues have produced a high carbonate bone in rats by administering a high calcium diet. In these rats, the carbonate to phosphate ratio of the blood is elevated, and the elevation is reflected in the elevation of the carbonate to phosphate ratio of the bone. The mechanism of this process is probably, at least in part, an exchange of carbonate for surface phosphate, a process described as heteroionic exchange by Neuman *et al.* *In vitro* studies have shown a linear increase in the percentage of CO_2 in the solid phase (synthetic hydroxylapatite or powdered bone mineral) with increasing ratio of total carbonate to total phosphate in solution. This is a competitive exchange that has been described by the following equation:

$$\text{Bone: } HPO_4 + CO_2 = \text{Bone: } CO_2 + HPO_4^{--}$$

The classical solubility studies of Hastings, Sendroy, and their colleagues of solutions in contact with solid calcium carbonate, solid "tertiary calcium phosphate," and mixtures of these two solids, for the first time gave insight into the relations of the concentrations of calcium, phosphate, bicarbonate, and hydrogen ions under conditions near those existing in the body. One of the interesting phenomena described was the ability of solid calcium phosphate to remove carbon dioxide from a solution in contact with a mixture of solid $CaCO_3$ and calcium phosphate. I should like to hear Doctor Hastings discuss the relation between the CO_2 removed from solution and the calcium removed simultaneously. To give him some data from his earlier work, two examples will be cited.

For the first, see Hastings *et al.*,* where, in table 12, on page 760, a system is described that contained solid calcium carbonate in contact with an aqueous solution of sodium citrate saturated for 20 hours at 38°C. with CO_2 at a pressure of 25 mm. The solution was separated from the solid, analyzed, then shaken with basic calcium phosphate, and again analyzed. Before the calcium phosphate treatment the solution contained 5.84 mM of calcium per kg. of water (per liter); after treatment, 3.78 mM/l. Thus, the solution lost 2.06 mM/l. The carbonate content expressed as total CO_2 was 12.65 mM/l before calcium phosphate treatment and 8.63 mM/l afterward; the solution lost 4.02 mM/l.

* Hastings, A. B., C. D. Murray & J. Sendroy, Jr. 1927. Studies of the solubility of calcium salts. I. The solubility of calcium carbonate in salt solutions and biological fluids. J. Biol. Chem. **71**: 723.

Therefore, one calcium disappeared for each two CO_2's, or, on an empirical basis, $Ca(HCO_3)_2$ was removed from solution. The initial pH was 7.3, the final pH 7.0; it is hard to attribute such a change to loss of CO_2 to the atmosphere.

The second example, (see Sendroy *et al.**), is taken from table 8 on page 818 of the quoted reference, experiment No. 8. A solution initially containing a high calcium concentration but no phosphate was saturated at 38°C. with a mixture of solid $CaCO_3$ and basic calcium phosphate. Initially, the calcium concentration was 7.76 mM/l and finally 1.33 mM/l; the solution lost 6.43 mM/l of calcium. The initial CO_2 content was given as a HCO_3^- concentration of 30.27 mM/l; the final concentration was 17.64 mM/l; thus the solution lost 12.63 mM/l of bicarbonate. Again the material removed apparently had approximately the composition of $Ca(HCO_3)_2$.

Despite the obvious interpretation that the CO_2 of bone may be deposited as the bicarbonate, this is not the case. Using a new infrared technique on solid bone, Underwood, Toribara and Neuman† have recently shown by direct measurement that essentially all of the CO_2 of bone is present as carbonate and that an undetectable amount of bicarbonate (if any) exists. While this answers the very important question of the nature of bone carbonate, it raises many questions concerning the mechanism of carbonate deposition.

Doctor Franklin C. McLean (*Department of Physiology, University of Chicago, Chicago, Ill.*): With reference to *phosphatase*, I am happy to note that, for the first time in years, it has been possible to sit through a conference on mineralized tissues without having to discuss the role of alkaline phosphatase in calcification. I have long subscribed to the belief that this enzyme is not required to provide phosphate ions for the bone salt, but that it probably assists in making the matrix of bone and of cartilage calcifiable. I hope that the absence of arguments to the contrary at this time means that this ghost is laid.

Chelation. There are two subjects which have not had the attention they deserve. Both have been mentioned by Doctor Hodge, but neither topic has been discussed. The one is the possibility that resorption of the mineral portion of bone is accomplished by the mechanism of chelation, a special form of complex formation. Until a few years ago, it was believed that the bone salt could be quickly and completely dissolved only in an acid medium. Now it is a common laboratory procedure to decalcify bone in a neutral or even in an alkaline medium containing a chelating agent, of which the prototype is Versene. It may be assumed that any solution containing a compound having a greater affinity for calcium than does the bone salt will be able to dissolve the mineral of bone. Moreover, there is a strong probability that the bone mineral is so dissolved *in vivo*, during the resorption of bone.

I was able to show, some nineteen years ago (unpublished observations), that the calcium in transit from bone to egg shell in the blood of laying hens is either not in equilibrium with the calcium ions of the blood, or that it is present in a very poorly dissociated complex. I offer as a working hypothesis the proposal that the bone salt is dissolved by complex formation, the nature of the com-

* Sendroy, J., Jr. & A. B. Hastings. 1927. Studies of the solubility of calcium salts. II. The solubility of tertiary calcium phosphate in salt solutions and biological fluids. J. Biol. Chem **71**: 783.

† Underwood, A. L., T. Y. Toribara & W. F. Neuman. 1955. An infrared study of the nature of bone carbonate. J. Am. Cancer Soc. **77**: 317.

pound assisting in its solution being as yet wholly unknown. Resorption of bone would then depend upon a combination of this action with solution of the bone matrix with the aid of a proteolytic enzyme. Perhaps both mechanisms are mediated by osteoclasts.

Homeostasis. The second subject to which I wish to call attention is that of the dual role of bone in the homeostatic regulation of the calcium ion concentration of the blood. I need not dwell on the probability that the fluids of the body are in some sort of dynamic equilibrium with the bone mineral. Moreover, there is now ample reason to believe that a part of the mineral, perhaps as much as 5 to 15 per cent, is in a labile form, and readily soluble in the body fluids. There remains to be made clear the interrelation of such a dynamic equilibrium with the action of the parathyroid hormone in maintaining the calcium ion concentration of the blood plasma at a constant level.

We have had strikingly illustrated, in Doctor Munson's presentation, the extreme rapidity with which the calcium ion level in the plasma falls after destruction of the parathyroid glands. Equally important is the fact that the fall is self limited. Even after parathyroidectomy the calcium ion concentration is regulated, but at a lower level. I submit that the lower level is that maintained by the equilibrium between the ions of the bone salt in the solid and liquid phases, and that the higher or normal level requires the action of the parathyroid hormone on bone. I would speculate that while solution of the labile portion of the bone mineral is accomplished readily and rapidly by simple chemical processes, the action of the parathyroid hormone is required to dissolve the stable crystal lattice. That this is accomplished by means of chelation, under the influence of the hormone, is also an attractive hypothesis.

Doctor Arthur Lindenbaum (*Division of Biological and Medical Research, Argonne National Laboratory, Chicago, Ill.*): In regard to the remark of Doctor Hastings on the supersolubility of calcium phosphate in serum, I should like to point out that in the treatment of hard water a somewhat analogous situation can be produced. This is a phenomenon known as "threshold action"* in which relatively large amounts of calcium can be held in solution by small amounts of condensed phosphates, such as pyrophosphate, tripolyphosphate, *etc.* Only small amounts of phosphates, around 10^{-5} M are needed, and the ratios of calcium held in solution to the added condensed phosphate may be as high as 100 to 300. These phosphates are thought to exert charge efforts which stabilize the calcium micelles and hinder crystal growth. It may be that a physiological counterpart of this phenomenon exists in the circulation.

Doctor Julius Sendroy, Jr. (*Naval Medical Research Institute, Bethesda, Md.*): The question has repeatedly arisen as to whether there is or is not an equilibrium between bone and plasma or other body fluids. May not some of our difficulties be resolved, if we bear in mind the fact, known to all of us, that in physiology there are few, if any, equilibria in the physicochemical sense of the term? What we do have are living systems maintained or operating in a *steady state* more or less removed from true equilibrium. This concept of biochemistry, often forgotten or ignored, may be of considerable help to us in such prob-

* Topley, B. 1949. Quart. Rev. Chem. Soc. 3(4): 345.
Martell, A. E. & M. Calvin. 1952. Chemistry of the Metal Chelate Compounds : 491.

lems as the reconciliation of *in vitro* and *in vivo* results in bone metabolism and calcification.

Doctor Allen Gee (*National Bureau of Standards, United States Department of Commerce, Washington, D. C.*): In organizing his solubility data on hydroxyapatite from many sources, Doctor Hodge found some interesting correlations. Some of his own solubility data (such as 1.6 mM Ca and 0.1 mM P at pH 6.5) point out an important factor frequently neglected. The excess of the equivalents of calcium over phosphorus in solution means that other anionic impurities are present. Since these impurities are of the order of the hydroxyapatite solubility, their presence cannot be ignored. This may mean that an adsorption phenomena is important even with very pure hydroxyapatite. A closer examination of overlooked factors, such as this, may explain why the solubility increases with the amount of solid phase and may eventually lead to a satisfactory expression for a solubility product of hydroxyapatite.

Doctor Leonard F. Bélanger (*Department of Histology, University of Ottawa, Ottawa, Ont., Canada*): By comparing the intense entry of S^{35} into enamel with the absence of picture over the epidermis and hair, it is considered that the S^{35} injected as sulfate is retained in the enamel as an organic form of sulfate, presumably as a mucoprotein rather than taking part in the synthesis of keratin. It is recalled that a British investigator* has already claimed the existence of a mucoprotein in the enamel on the basis of chemical analysis. The basophilia of the pre-enamel layer is in favor of the presence of such an acid substance. This mucoprotein, as it appears by autoradiographs of subsequent periods, is rapidly metabolized and little is found in acidophilic maturing enamel. Its role may be to prevent mineralization of the pre-enamel until the complete thickness of this substance has been achieved (J. Dental Research, in press).

Doctor Karl Meyer (*College of Physicians and Surgeons, Columbia University, New York, N. Y.*): I wish to make it clear that I have no personal experience with the problem of calcification or with the ground substance of bone. I believe, however, we cannot accept the statement that a collagen complex of chondroitin sulfate is the basis of the mechanism of calcification. First, there is more than one chondroitin sulfate; in fact, we distinguish three chondroitin sulfates. Recently, we found a new sulfated mucopolysaccharide not related at all to chondroitin sulfate, a polymer of a disaccharide composed of N-acetylglucosamine and galactose. We found this new complex in cornea. The chondroitin sulfates occur in cartilage and in all connective tissue. We have to explain why these ordinarily do not calcify and bone does. From the literature and from the discussion here, I think a good argument has been made for the role of the ground substances in calcification. If we want to understand this role, I should certainly look for differences in the nature or quantity of the substances present in bone and absent in other collagenous organs. There is one other point I should like to stress here. Apparently, it has been tacitly assumed that the protein in collagenous tissue which forms a complex with the mucopolysaccharides is collagen. In my opinion, this has not been proved. In tendon and heart valves, for example, we know the chondroitin sulfates are not bound to collagen but to a protein rich in aromatic amino acids. I think the proteins

* Pincus, P. 1949. Brit. Dental J. 86: 226.

of hyaline cartilage ought to be investigated for the presence of proteins distinct from collagen. One such protein, for example, which occurs in hyaline cartilage in concentrations higher than in other mesodermal tissues, is lysozyme. Another question which merits study is the question whether the collagens of cartilage, of bone, and of other collagenous tissues are identical or not. There are certainly tremendous differences in the extractability of the collagens from different organs. In other words, we do not know the chemical nature of the proteins nor of the polysaccharides in bone or the changes they undergo in the zones of calcification.

The suggestion of a phosphorylated hexosamine or phosphorylated mucopolysaccharide, proposed by Doctor Neuman, is interesting. The stability to, and hydrolysis of, such a phosphate ester is surprising and one would certainly want such a compound isolated and chemically characterized. In most mucopolysaccharides, the stability of the hexosaminidic bond is quite remarkable and, in chondroitin sulfate, for example, the estersulfate group is hydrolyzed much more rapidly than either the hexosaminidic or glucuronidic groups.

The last problem I might comment on is the problem of the sulfatation of the mucopolysaccharides. The sulfate uptake of the epiphysis as well as of other structures is certainly impressive and convincing. We should like to know how the sugars are sulfated, when they are sulfated, and how are they degraded or disposed of, for example, when cartilage ossifies. To take the last question first, there is no evidence whatever for the presence of sulfatases in animal tissues capable of attacking carbohydrate ester sulfates, in contrast to the well-known phenol-sulfatases, which are widely distributed. Testicular hyaluronidase does hydrolyze chondroitin sulfate of cartilage and one of the two chondroitin sulfates present in loose connective tissue, but the oligosaccharides thus produced are still fully sulfated. Why hyaluronic acid *in vivo* is not sulfated, I do not know. A fraction recently isolated by us appears to be chondroitin, that is, the desulfated, or rather not yet sulfated, chondroitin sulfate. We interpret this finding as indicating that the polysaccharide chain is first polymerized and then sulfated and not the other way around. We have begun looking for this fraction in growing bone.

Doctor Martin Silberberg (*Washington University, St. Louis, Mo.*): With reference to the papers of Doctor Wilkins and Doctor Shaw, I should like to point out that the skeletal findings in humans suffering from hormonal imbalances agree in many respects with those produced in animals by removal of endocrine glands or by administration of hormones. Among the hormones one may distinguish growth promotors and growth inhibitors. The multiplicity of the growth factors should be re-emphasized as well as the fact that substances that promote skeletal growth also promote skeletal development and aging. Anterior hypophyseal hormone stimulates growth of cartilage, but this is usually followed by acceleration of skeletal development and aging. Thyroid hormone slightly stimulates growth of cartilage but exerts a more conspicuous effect on skeletal development. Thyroidectomy in general delays skeletal development, and after "radiation-induced thyroidectomy" with I^{131}, a peculiar lesion of the meniscus is noted that may be related to epiphyseal dysgenesis seen in hypothyroid children. Small doses of testosterone enhance

skeletal growth and development. Large doses of the hormone inhibit growth but promote skeletal development and aging as do estrogens and adrenal cortical steroids. Hormones that act on the epiphyseal cartilage also affect the joints and thus acquire significance in the pathogenesis of arthritis. The metaphyseal changes seen in vitamin A deficiency bear some similarity to those found after thyroidectomy or after administration of estrogen. This similarity leads to the intriguing question of the interaction of hormonal and nutritional factors in osteogenesis.

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